

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**IMPROVEMENT OF DEPTH-SENSITIVE
OPTICAL SPECTROSCOPY AND IMAGING
WITH WAVEFRONT SHAPING AND
RADIOFREQUENCY TAGGING**

HSIEH CHAO-MAO

**School of Chemistry, Chemical Engineering and
Biotechnology**

2023

**IMPROVEMENT OF DEPTH-SENSITIVE
OPTICAL SPECTROSCOPY AND IMAGING
WITH WAVEFRONT SHAPING AND
RADIOFREQUENCY TAGGING**

HSIEH CHAO-MAO

**School of Chemistry, Chemical Engineering and
Biotechnology**

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2023

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

09 Jan 2023

.....

Date



[Hsieh Chao-Mao]

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

09 Jan 2023

.....

Date



[Prof. Duan Hongwei]

Authorship Attribution Statement

This thesis contains materials from 3 paper(s) published in the following peer-reviewed journal(s) in which I am listed as an author.

Chapter 3 is published as “Hsieh, C. M., Verma, M., & Liu, Q. (2019). Improving depth sensitive fluorescence spectroscopy with wavefront shaping by spectral and spatial filtering. IEEE Access, 7, 170192-170198.”

The contributions of the co-authors are as follows:

- A/Prof Liu Quan provided the initial project direction.
- I prepared the manuscript drafts. The manuscript was revised by A/Prof Liu Quan.
- I co-designed the study with A/Prof Liu Quan and performed all the laboratory work at the School of Chemistry, Chemical Engineering and Biotechnology. I also analyzed the data.
- I conducted experiments related to the article, including establishing optical setup, sample preparation, programming, signal measurement, and data analysis, in School of Chemistry, Chemical Engineering and Biotechnology.

Chapter 5 is published as “Hsieh, C. M., Ren, X., & Liu, Q. (2022). Feedback-based wavefront shaping for weak light with lock-in beat frequency detection. *Optics Letters*, 47(19), 5192-5195.”

The contributions of the co-authors are as follows:

- A/Prof Liu Quan provided the initial project direction.
- I prepared the manuscript drafts. The manuscript was revised by A/Prof Liu Quan.
- I co-designed the study with A/Prof Liu Quan and performed all the laboratory work at the School of Chemistry, Chemical Engineering and Biotechnology. I also analyzed the data.
- I conducted experiments related to the article, including establishing optical setup, sample preparation, programming, signal measurement, and data analysis in the School of Chemistry, Chemical Engineering and Biotechnology.

Chapter 6 is published as “Hsieh, C. M., Malik, M. O., & Liu, Q. (2023). High-speed feedback based wavefront shaping for spatiotemporal enhancement of incoherent light through dynamic scattering media. Optics Letters, 48(9), 2313-2316.”

The contributions of the co-authors are as follows:

- A/Prof Liu Quan provided the initial project direction.
- I prepared the manuscript drafts. The manuscript was revised by A/Prof Liu Quan.
- I co-designed the study with A/Prof Liu Quan and performed all the laboratory work at the School of Chemistry, Chemical Engineering and Biotechnology. I also analyzed the data.
- I conducted experiments related to the article, including establishing optical setup, sample preparation, programming, signal measurement, and data analysis in the School of Chemistry, Chemical Engineering and Biotechnology.

02 May 2023

.....
Date

NTU NTU NTU NTU NTU NTU NTU NTU
NTU NTU NTU NTU NTU NTU NTU NTU
NTU NTU NTU NTU NTU NTU NTU NTU
NTU NTU NTU NTU NTU NTU NTU NTU
.....
[Hsieh Chao-Mao]

Acknowledgments

I would like to take this opportunity to offer my deepest and sincerest gratitude to all people who have contributed to this dissertation.

I firstly would like to thank my thesis and research supervisor, Prof. Liu Quan, for his guidance and continuous support for the research and dissertation throughout my Ph.D. study. I learn a lot from his informative discussion about solving research problems with his valuable feedback. The 4-year study performed by my study could not have been finished without a great environment provided by him and his support. My gratitude extends to acknowledge my co-supervisor, Prof. Duan Hongwei, for his financial support and for helping me continue studying in the final year of my Ph.D. study. Special thanks to two thesis advisory committees – A/Prof. Manojit Pramanik and Prof. Peter Török for their valuable research and thesis suggestions.

Next, I want to thank our group members in the lab – Dr. Ren Xiaojing, Mohammad Malik, Dr. Zhang Yi, Dr. Kang Jian, Dr. Luo Yuemei, Dr. Bai Yanru, Dr. Clement Yuen, Dr. Kumar Varun, Dr. Ju Jian, Dr. Tian Yao and Su Weiming Joshua for offering their assistance and suggestions on both research experiments and daily life. I learned a lot and developed new knowledge in this field from work collaborations. They turned the lab into an enjoyable and friendly environment.

I also would like to thank the Nanyang Technological University (NTU) and School of Chemistry, Chemical Engineering and Biotechnology (CCEB) for the financial support with a scholarship and for providing an excellent environment with resourceful laboratory facilities and equipment to allow me to finish my

Ph.D. study. I also want to thank all the staff in CCEB for their support in administrative matters.

Most importantly, I would express my earnest gratitude to my family and friends for their help, emotional support, and accompany during my Ph.D. journey.

Hsieh Chao-Mao Jan 2023

Abstract

The dissertation describes a series of studies developing depth-sensitive optical spectroscopy assisted by wavefront shaping and radiofrequency tagging. Depth-sensitive optical spectroscopy preferentially detects optical spectra from different depths in layered samples, which plays a crucial role in many applications, such as the optical diagnosis of epithelial precancer and cancer. For depth-sensitive optical measurements, multiple light scattering effects and the requirement of axial scanning cause limit the performance of related techniques. First, multiple light scattering in a highly scattering sample, such as a tissue, significantly degrades the depth sensitivity to a subsurface target layer. Second, the performance of enhancement techniques such as wavefront shaping can be unstable in dynamic samples, for example, during *in vivo* measurements of tissues with blood flow right beneath. Third, axial scanning achieved by mechanical motion is time-consuming, especially for an extended depth range. To address these issues, three techniques, including “feedback-based wavefront shaping with lock-in method,” “FPGA-based AOD modulation,” and “radiofrequency tagging for depth-resolved imaging,” are developed to improve depth-sensitive optical spectroscopy.

In the first study in this dissertation (Chapter 3), spatial and spectral filtering can improve the performance of feedback-based wavefront shaping. The lack of intrinsic guide stars in tissues or tissue-like samples often leads to the poor enhancement of depth-sensitive Raman/fluorescence measurements (~20% in the past literature) from a target layer due to the contributions from the overlaying non-target layers. In this study, we demonstrate that spatial and

spectral filtering can significantly improve the performance of depth-sensitive fluorescence spectroscopy assisted by feedback-based wavefront shaping (using an SLM) in tissue-like scattering phantoms. The two filtering techniques work by effectively increasing the relative contribution from the target layer to the feedback signal during wavefront optimization through spatially and spectrally rejecting off-target fluorescence light, essentially similar to the role of time or coherence gating.

To speed up wavefront shaping for real applications, we explore using an AOD as a phase modulator instead of SLM in the second study (Chapters 4 and 5). The high refreshing rate and optical frequency shifts of the AOD provide unique advantages for wavefront shaping. It is well known that the signal-to-noise ratio is essential for feedback-based wavefront shaping. The technique can fail for low optical signals such as fluorescence and Raman signals or in a reflection setup because the trend in weak feedback signals can be easily overwhelmed by noise. To address this challenge, we develop a technique based on a single acousto-optic deflector (AOD) (Chapters 4 and 5) to create a signal with a selected beat frequency from optical signals that can serve as feedback, in which the phase distribution of various radio frequency components of the driving signal for the AOD is optimized for wavefront shaping. By shifting incident light frequency with the AOD, the feedback signal at a selected beat frequency can be measured with a high signal-to-noise ratio by a lock-in amplifier, thus enhancing weak target signals through highly scattering media. The lock-in beat-frequency detection method can significantly improve fluorescence imaging and Raman spectral measurements in a reflection setup and thus could be potentially used for *in vivo* measurements.

Feedback-based wavefront shaping can perform poorly for dynamic samples due to short correlation time. So far, most proposed methods for high-speed wavefront shaping can only enhance the intensity of coherent light but not incoherent light. Therefore, it is necessary to develop high-speed wavefront shaping to directly enhance incoherent light, such as fluorescence, which is essential in extending wavefront shaping to biomedical applications in which fluorescence spectroscopy/imaging is broadly adopted. For this purpose, we develop a technique based on a single acousto-optic deflector (AOD) with field-programmable gate array (FPGA) acceleration for spatiotemporal focusing within milliseconds (Chapter 6). With the digital time gating of the feedback signal, the spatiotemporal focusing of laser light with high contrast can be formed behind dynamic scattering media in milliseconds resulting in significant fluorescence enhancement. Furthermore, FPGA-based wavefront shaping is shown to effectively enhance fluorescence directly behind dynamic samples with short correlation times.

The final study (Chapter 7) is to improve the speed of depth-sensitive measurements by generating multiple foci along the axial dimension, with each depth encoded by a unique radio frequency. With remote focusing and multiple focal spots at different depths, each with a unique radiofrequency beat signal generated using a tilted mirror and an AOD, respectively, signals from different depths in a sample can be simultaneously measured with a fast single-pixel detector at high speed. Because the depth information is encoded in the RF signal, we can reconstruct depth-relevant information from the data measured by the single-pixel detector. This technique dramatically increases the speed of depth-sensitive spectroscopy without involving mechanical movement. This method

can complement the current methods for sequential depth-dependent measurements, involving a piezo actuator, an electrically tunable lens, and a TAG lens, by increasing effective exposure time and improving the overall measurement throughput.

Table of Contents

Abstract	I
Table of Contents	V
List of Figures	IX
Glossary of Acronyms	XII
1. Introduction	1
1.1 Background and Motivation	1
1.1.1 <i>Depth-Sensitive Optical Spectroscopy</i>	1
1.1.2 <i>Multiple Light Scattering in Thick Samples</i>	2
1.1.3 <i>Optical Measurement Techniques for Overcoming the Issue of Multiple Scattering</i>	3
1.2 Objectives	9
1.2.1 <i>Aim 1: To Improve the Depth Sensitivity of Measured Optical Spectra with Feedback-based Wavefront Shaping</i>	9
1.2.2 <i>Aim 2: To Achieve Real-Time Feedback-Based Wavefront Shaping for Dynamic Samples</i>	10
1.2.3 <i>Aim 3. To Accelerate Depth-Sensitive Optical Measurements</i>	12
1.3 Outline of Dissertation	13
2. Wavefront Shaping	15
2.1 Principle of Wavefront Shaping	16
2.2 Three Types of Wavefront Shaping Techniques	19
2.3.1 <i>Measurements of the Transmission Matrix (TM)</i>	19
2.3.2 <i>Optical Phase Conjugation (OPC)</i>	21
2.3.3 <i>Feedback-Based Wavefront Shaping</i>	23
2.3 Application of Wavefront Shaping in Biomedical Optics	24
2.3.1 <i>Optogenetics and Optical Tweezer</i>	24
2.3.2 <i>In vivo Deep Tissue Imaging</i>	25
2.4 Feedback-Based Wavefront Shaping for Depth-Sensitive Spectroscopy	26
2.4.1 <i>Feedback Signal</i>	26
2.4.2 <i>Optimization Algorithm</i>	27
2.4.3 <i>Limitations of Feedback-Based Wavefront Shaping</i>	30
2.4.4 <i>Improvement of Feedback-Based Wavefront Shaping</i>	31
3. Improving Depth-Sensitive Fluorescence Spectroscopy with Wavefront Shaping by Spectral and Spatial Filtering	32

3.1. Introduction	32
3.2. Principle and Methodology	34
3.2.1. <i>Experimental Setup</i>	34
3.2.2. <i>Phantom Preparation</i>	36
3.2.3. <i>SLM Modulation</i>	37
3.2.4. <i>Enhancement Factor and Emission Ratio</i>	37
3.2.5. <i>Spectral and Spatial Filtering</i>	38
3.3. Experimental Results	39
3.3.1. <i>Wavefront Shaping with Spatial Filtering</i>	39
3.3.2. <i>Wavefront Shaping with Spectral Filtering</i>	41
3.4. Discussion	44
3.4.1. <i>Effects of Spatial and Spectral Filtering</i>	44
3.4.2. <i>Signal-to-Noise Ratio (SNR)</i>	44
3.4.3. <i>Photobleaching Artifact</i>	45
3.4.4. <i>Continuous Sequential Algorithm</i>	46
3.4.5. <i>Homogenous Samples and Discrete Targets</i>	46
3.5. Conclusion	47
4. Wavefront Shaping with AOD Modulation	49
4.1 Introduction	49
4.2 Principle and Methodology	50
4.2.1 <i>Phase/amplitude Modulation of Light with Acoustic Wave</i>	50
4.2.2 <i>Generation of Multiple Diffracted Laser Beams</i>	53
4.2.3 <i>Feedback Signal Selection</i>	54
4.2.4 <i>Sample Preparation</i>	55
4.3 Experimental Results	57
4.3.1 <i>Focusing Laser Light through TiO₂ Phantoms</i>	57
4.3.2 <i>Enhancement Factor and Laser Power</i>	59
4.3.3 <i>Phase and Amplitude Modulation</i>	60
4.3.4 <i>Comparison with SLM Modulation</i>	62
4.3.5 <i>Enhancement of Fluorescence Spectra</i>	63
4.3.6 <i>Fluorescence Enhancement: Laser-Guided vs. Fluorescence-Guided Wavefront Shaping</i>	64
4.4 Discussion	67
4.5 Conclusion	69
5. Feedback-based Wavefront Shaping for Weak Light with Lock-in Beat Frequency Detection	70

5.1. Introduction	70
5.2. Principle and Methodology.....	73
5.2.1 Principle	73
5.2.2 Experiment Apparatus	75
5.3. Experimental Results	77
5.3.1 Spatiotemporal Focusing (Laser Speckles).....	77
5.3.2 Wavefront Shaping with Lock-in Detection for Laser Speckle in Transmission Setup	80
5.3.3 Comparison in Enhancement Factor of Laser Light Transmitted through a Scattering Media among Different Feedback Strategies.....	81
5.3.4 Fluorescence Enhancement in Transmission Setup	83
5.3.5 Fluorescence Enhancement in Reflection Setup	85
5.3.6 Raman Signal Enhancement in Reflection Setup	89
5.4. Discussion	93
5.4.1 Comparison of Spatiotemporal Focusing and Lock-in Detection	93
5.4.2 Memory Effect.....	95
5.4.3 Improvement of Raman Signal Measurement	96
5.5. Conclusion.....	96
6. Accelerating Feedback-Based Wavefront Shaping with FPGA Control and AOD Modulation	97
6.1 Introduction	97
6.2 Principle and Experimental Apparatus.....	100
6.2.1 Principle of FPGA-Based Wavefront Shaping	100
6.2.2 Experimental Setup.....	106
6.2.3 Dynamic Samples	108
6.3 Experimental Results	110
6.3.1 Continuous Wavefront Shaping vs. Single-time Wavefront Shaping	110
6.3.2 Continuous Wavefront Shaping on Dynamic Samples for Laser Speckle Enhancement.....	111
6.3.3 Continuous Wavefront Shaping on Dynamic Samples for Fluorescence Enhancement	115
6.3.4 Ex Vivo Measurements.....	119
6.4 Discussion	127
6.5 Conclusion.....	129
7. Fast Depth-Resolved Imaging Based on Radiofrequency Tagging	130
7.1 Introduction	130
7.2 Experimental Principle and Apparatus.....	132

7.2.1	<i>Principle of AOD-Depth Scan</i>	132
7.2.2	<i>Experiment Apparatus</i>	135
7.3	Experimental Results	139
7.3.1	<i>Generation of Multiple Laser Focal Spots</i>	139
7.3.2	<i>Simultaneous Measurements of Depth-Resolved Signals</i>	141
7.4	Discussion	144
7.3.3	<i>Resolution and the Depth Range</i>	144
7.3.4	<i>Potential Improvement</i>	145
7.5	Conclusion	146
8.	Conclusions and Future Directions	147
8.1	Conclusions	147
8.1.1	<i>Feedback-Based Wavefront Shaping with Different Strategies for Improvement</i>	147
8.1.2	<i>Simultaneous Measurement of the Signal from Different Depth</i>	148
8.2	Future Directions	149
8.2.1	<i>Lock-in Detection for Raman Measurements</i>	149
8.2.2	<i>Multi-channel Lock-in Detection for Generating Spatiotemporal Focusing</i>	151
8.2.3	<i>FPGA Acceleration for Wavefront Shaping with Lock-in Detection</i>	153
Author Publications		156
References		158

List of Figures

Fig. 1.1. Multiple light scattering caused by highly scattering media.	3
Fig. 2.1. Illustration of the continuous sequential algorithm (CSA).	28
Fig. 2.2. Illustration of the Optimal Steepest Ascent (OSA) algorithm.	29
Fig. 3.1. Experimental setup.	35
Fig. 3.2. Enhancement of emitted fluorescence with and without a 200- μm pinhole.	41
Fig. 3.3. Effect of spatial filtering using pinholes on the performance of wavefront optimization.	42
Fig. 3.4. Effect of spectral filtering using band-pass filters on the performance of wavefront optimization.	43
Fig. 4.1. The 1D diffracted light pattern is generated by a single AOD driven by the superposition of 41 RF components (60 – 100 MHz).	53
Fig. 4.2. The first-order diffraction efficiency with different numbers of driving frequencies in an AOD (AODF 4090-7; G&H).	54
Fig. 4.3. Relation between the inverse transmission and the sample thickness of a TiO_2 phantom.	56
Fig. 4.4. Focusing light through a scattering TiO_2 phantom.	58
Fig. 4.5. Effects of degrees of freedom and laser power on enhancement factor.	60
Fig. 4.6. Comparison of phase and phase-amplitude modulation.	61
Fig. 4.7. Comparison of the SLM and AOD modulation with different incident powers.	62
Fig. 4.8. Emission spectra of.	64
Fig. 4.9. Laser-guided vs. fluorescence-guided wavefront shaping for fluorescence enhancement.	66
Fig. 4.10. 2D camera image of the target region	68
Fig. 5.1. Experimental setup of feedback-based wavefront shaping with lock-in detection of weak light.	76
Fig. 5.2. Setup for fluorescence detection after transmission through a scattering media.	76
Fig. 5.3. Performance of wavefront shaping when using the sum of magnitudes of all beat-frequency components as the feedback.	79
Fig. 5.4. Comparison of temporal focusing between strong and weak feedback after wavefront shaping.	79
Fig. 5.5. Feedback in wavefront shaping in the (a) time domain and (b) frequency domain before and after wavefront shaping using AOD.	80
Fig. 5.6. Enhancement factor as a function of detected light power for three strategies different in the choice of feedback.	83
Fig. 5.7. 2D imaging of quantum dots clusters with different strategies.	85
Fig. 5.8. Wavefront shaping with lock-in detection for fluorescence enhancement.	87
Fig. 5.9. Beat signals from fluorescence with or without multiple scattering.	88

Fig. 5.10. Optical setup (detection part) for Raman scattering enhancement.	91
Fig. 5.11. Wavefront shaping with lock-in detection for Raman scattering enhancement.	91
Fig. 5.12. Comparison of feedback wavefront shaping using DC or AC signals as the feedback.	92
Fig. 5.13. MATLAB simulation.	94
Fig. 6.1. Temporal focusing generated by AOD modulation	103
Fig. 6.2. Focal spot is formed behind a diffuser after wavefront shaping.	104
Fig. 6.3. Time chart of feedback-based wavefront shaping using FPGA with 32 degrees of freedom.	106
Fig. 6.4. Schematic of the setup for FPGA-based wavefront shaping for laser speckle and fluorescence measurement.	107
Fig. 6.5. Correlation time determination of a dynamic sample.	109
Fig. 6.6. Effect of continuous wavefront shaping on a dynamic sample.	110
Fig. 6.7. Continuous wavefront shaping for a static sample moving on a motorized stage.	112
Fig. 6.8. Temporal focusing through scattering samples with different correlation times.	114
Fig. 6.9. Performance of wavefront shaping to focus light behind dynamic intralipid phantoms with different correlation times.	115
Fig. 6.10. Temporal focusing for fluorescence signal without through scattering media.	116
Fig. 6.11. Performance of wavefront shaping to enhance fluorescence signals measured behind intralipid phantoms on a motorized stage with different correlation times.	117
Fig. 6.12. 2D imaging of fluorescent particles behind dynamic scattering media with different correlation times after wavefront shaping.	118
Fig. 6.13. Chicken breast tissue slices with different thicknesses and a commercial diffuser for comparison.	120
Fig. 6.14. Laser speckles are generated behind chicken tissues with different thicknesses.	121
Fig. 6.15. Performance of wavefront shaping for enhancing laser light behind chicken breast tissues with different correlation times.	122
Fig. 6.16. Optimized signal intensity after wavefront shaping behind a chicken breast tissue as a function of correlation time.	122
Fig. 6.17. Feedback-based wavefront shaping for temporal focusing with 1-MHz filtering.	125
Fig. 6.18. Comparison of two methods using the whole signal or filtered 1-MHz signal as feedback for wavefront shaping.	125
Fig. 6.19. One-dimensional profile of fluorescent particles behind chicken breast tissues with different correlation times after wavefront shaping.	126
Fig. 7.1. Remote focusing with a steering mirror.	133
Fig. 7.2. Modulation of focal depth with a tilt mirror for generation of focus spots at different depths.	134
Fig. 7.3. Generation of depth-dependent beat signals.	135

Fig. 7.4. Schematic of the optical setup for generating multiple focal spots with different depths by remote focusing.	137
Fig. 7.5. Beat signal generation by the interference between two arms.	138
Fig. 7.6. Measurement of the beat signal by APD.	138
Fig. 7.7. Profile of multiple laser focal spots. Driving frequencies (75, 80, 85, 90, 95 MHz) are used to generate five laser focus spots for demonstration.	140
Fig. 7.8. Fluorescent profile at different depths from the focusing laser light with the different beat frequencies.	141
Fig. 7.9. Reconstructing fluorescent particle images in the Y-Z plane.	143
Fig. 7.10. 2D image of multiple fluorescent particles.	144
Fig. 7.11. Reflection setup for simultaneous depth-sensitive measurements.	145
Fig. 8.1. Feedback-based wavefront shaping with lock-in detection for Raman signal enhancement by using common Raman peak as feedback.	150
Fig. 8.2. Preliminary study of multi-channel lock-in method for temporal focusing.	152
Fig. 8.3. Proposed method for spatiotemporal focusing of a weak signal using multi-channel lock-in amplifier and FPGA acceleration.	152
Fig. 8.4. Closed loop system of feedback-based wavefront shaping under different conditions.	155
Fig. 8.5. Strategies of wavefront shaping in different conditions based on the SNR of the feedback signal.	155

Glossary of Acronyms

AC: Alternative current

ADC: Analog-to-digital converter

AO: Adaptive optics

AOD: Acousto-optic deflector

AOFS: Acousto-optic frequency shifters

APD: Avalanche photodetector

CCD: Charge-coupled device

CGH: Computer-generated holography

CSA: Continuous sequence algorithm

CTIS: Computed tomography imaging spectrometer

DAC: Digital-to-analog converter

DC: Direct current

DOF: Degrees of freedom

DOPC: Digital optical phase conjugation

DMD: Digital Micromirror Devices

DM: Dichroic mirror

DM: Deformable mirror

EPR: Enhanced permeability and retention

ETL: Electrically tunable lens

FDM: Frequency-division-multiplexed

FDPM: Frequency-Domain Photon Migration

FEST: Frequency-encoded spatiotemporal

FOV: Field of view

FPGA: Field-programmable gate array

FWHM: Full width at half maximum

GA: Genetic algorithm

GLV: Grating light valve

GPC: Generalized phase contrast

HWP: Half-wave plate

LUT: Look-up table

MEMS-SLM: Microelectromechanical systems spatial light modulator

MPM: Multi-photon microscopy

NA: Numerical aperture

OCT: Optical coherence tomography

OPC: Optical phase conjugation

OSA: Optimal steepest ascent

PA: Partitioning algorithms

PDMS: Polydimethylsiloxane

PMT: Photomultiplier tube

QWP: Quarter-wave plate

RF: Radio frequency

SA: Simulated annealing

SBR: Signal-to-background ratio

sCMOS: Scientific complementary metal oxide semiconductor

SERS: Surface-enhanced Raman scattering

SFDI: Spatial frequency domain Imaging

SLM: Spatial light modulator

SNR: Signal-to-noise ratio

TM: Transmission matrix

TROVE: Time reversal of variance-encoded light

TRUME: Time-reversed ultrasound microbubble encoded

TRUE: Time-reversed ultrasonically encoded light

WD: Working distance

WS: Wavefront Shaping

Chapter 1

Introduction

1.1 Background and Motivation

1.1.1 *Depth-Sensitive Optical Spectroscopy*

In biomedical optics, light measurements in imaging, microscopy, and spectroscopy can be used for noninvasive tissue diagnosis, for example, to detect diseases in their early stage. The progress of diseases is critical to early diagnosis [1]. For layered tissues like skin tissue, where the depth distribution of chromophores is essential, optical measurements from different depths could provide a means for diagnosing skin cancer and monitoring its development. For this purpose, Raman spectroscopy [2] is a suitable noninvasive technique for investigating the chemical composition of tissues to separate lesion tissues from normal tissues based on changes in molecular characteristics [3-5]. Depth-sensitive optical spectroscopy [6, 7] is the technique to measure and analyze optical spectra from different depths in layered tissues. Several depth-sensitive optical spectroscopy techniques have been proposed, including fiber-based depth-sensitive optical spectroscopy [6, 8] for fluorescence measurements and spatial offset Raman spectroscopy [9, 10] for Raman measurements. Although depth-sensitive optical spectroscopy significantly improves the measurements of the signal from a specific depth, the penetration depth and the depth sensitivity of

depth-sensitive measurements degrade substantially with the increasing depth in a thick tissue when the effect of multiple light scattering becomes dominant.

1.1.2 Multiple Light Scattering in Thick Samples

Unlike a transparent medium in which light can travel through it along a straight pathway without distortion, light in a highly scattering medium will undergo multiple scattering, which induces difficulty in the precise measurements of a target inside the medium (**Fig. 1.1**). The reduced scattering coefficient (μ_s') and absorption coefficient (μ_a) characterize the scattering and absorption property, respectively. In most biological samples, the interaction between light and the sample is dominated by multiple scattering instead of absorption [11]. Light scattering in tissues is caused by the interaction with particles close to the wavelength of incident light in size, i.e., Mie scattering [12]. The conversion of ballistic light to scattered light changes the direction of light propagation, causing the loss of spatial information. With the effect of multiple scattering, light is strongly defocused in a scattering sample with the thickness of one transport mean free path (l_t), which induces degradation in the spatial resolution and penetration depth of optical imaging as well as in the depth sensitivity of optical spectroscopy. For tissues such as chicken breast tissue with 690- μm thickness, each transmitted photon can undergo up to 26 scattering events [13]. Furthermore, the number of scattering events can go up to 40 for 1-mm chicken breast [14, 15]. In these conditions, ballistic light is too weak to be detected. It is worth noting that visible light (400-700 nm), commonly used in biomedical studies, has much stronger scattering than near-infrared light (NIR), significantly reducing the penetration depth in tissue diagnosis. In short, multiple light scattering limits the

applications of optical-based techniques in thick biological samples for optical diagnosis, especially in the visible region.

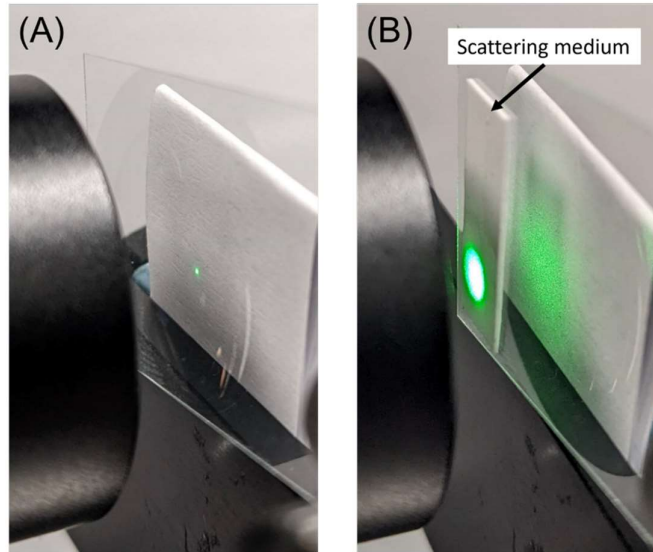


Fig. 1.1. Multiple light scattering caused by highly scattering media. (A) Light can be focused onto a small spot by an objective. (B) Laser speckles are generated from defocused light when convergent light encounters a scattering medium.

1.1.3 Optical Measurement Techniques for Overcoming the Issue of Multiple Scattering

So far, many techniques have been published to deal with signal measurement in disordered media such as tissues by overcoming the multiple scattering to retrieve the valuable signal from the specific target in the thick tissues.

- *Optical Coherence Tomography (OCT)*

OCT [16, 17] uses coherence gating to reject multiple scattered lights and can image up to 2 mm. The coherence gating is performed using low coherence light

such that interference only occurs when the optical paths of two light beams are matched within the short coherence length. However, singly scattered light in a sample is too weak to detect for a deep target, limiting the penetration depth. Hence, its principle does not apply to fluorescence spectroscopy or Raman spectroscopy.

- ***Multi-Photon Microscopy (MPM)***

For multi-photon microscopy [18, 19], nonlinear fluorescence excitation is based on the generation of a photon with higher energy upon the simultaneous absorption of multiple photons with lower energy, and the process is triggered by the combined energy from multiple photons that meet the requirement of electronic transition from the ground state to an excited state. A high-power pulsed laser is usually used as the light source to provide the required high energy in a short time to generate high photon density. In the optical design, the focal spot of the objective with high-density photons, both spatially and temporally, is the place leading to multiphoton excitation. Therefore, a high signal-to-background ratio can be achieved since fluorophore excitation is confined to the small surrounding region of the focal point.

In biological applications, a near-infrared light source provides photons with low energy while the typical fluorescence excitation wavelength is in the visible region. Compared to visible light, near-infrared light has a greater penetration depth since the scattering strength of tissue is usually inversely proportional to the wavelength. Therefore, multiphoton microscopy has the advantage of penetration depth for endogenous chromophores. Another advantage is that, with the use of a pulsed laser in a scattering sample, scattered light has a

low possibility of meeting the requirement of nonlinear exaction in space or time. As a result, most fluorescence will be only triggered by unscattered light (ballistic light). Hence, even with multiple scattering inside the tissue, fluorescence excitation is still limited to a small spatial point. The maximum penetration depth is determined by ballistic light, which decreases with depth.

- ***Time Gating***

When a laser pulse travels in a scattering medium, a small portion of photons travels without being scattered or undergo only a couple of scattering events, often called ballistic photons. In contrast, others get diffused after multiple scattering. The diffused photons spend more time in the medium before exiting than ballistic photons. As a result, the diffused photons usually reach a detector later than the ballistic photons. The time-gating technique [20-22] is developed to select photons based on the time of arrival to a photodetector. The selection of the photons can isolate the ballistic light or singly scattered photons by rejecting multiply scattered photons. Ballistic photons can achieve a higher spatial resolution [23, 24] when used for imaging if the SNR is not an issue. The technique is also suitable for isolating the Raman scattering from the fluorescence background due to their different lifetimes [25]. However, the method is unsuitable for fluorescence and Raman measurement due to weak signals.

- ***Spatial Frequency Domain Imaging (SFDI)***

Scattering and absorption coefficient are the two critical optical parameters for analyzing the optical properties of biological samples. Scattering dominates diffusion reflectance in a deeper layer for a turbid sample with heterogeneous

components. Therefore, separating the absorption and scattering effect in the detected signal from the sample is crucial to retrieving optical properties that could be useful for clinical application. SFDI [26] can rapidly and quantitatively measure the reduced scattering coefficient (μ_s') and absorption coefficient (μ_a) from Monte Carlo simulation or diffusion approximation in a wide field of view with low-cost equipment [27, 28]. The capability of SFDI to separate the absorption and scattering effects in light propagation enables it to apply in some clinical applications, such as measuring the concentrations of some components (e.g., concentration of oxygenated hemoglobin (Hb)) in tissues. Also, SFDI has the resolution of the submillimeter and depth of penetration of the centimeter scale for clinical requirements [26]. For the rapid large-field measurement, the structured illumination used in the SFDI speeds up the process by imaging in a large field without the raster scanning step. Furthermore, the speed can be increased close to real-time measurement by combining with another technique, such as computed tomography imaging spectrometer (CTIS), to avoid the spectral scanning process [29] or using high spatial frequency illumination to do the wide field imaging [30]. The variation in spatial frequency can slightly modify the probing depth. While this technique deals with multiple scattering by fitting to the Monte Carlo results or diffusion theory, it cannot yield a high spatial resolution.

- *Frequency-Domain Photon Migration (FDPM)*

Frequency Domain Photon Migration (FDPM) [31] is a technique that tracks photon migration through tissue with transport theory since light propagation is the wave function that comprises scattering and absorption. Like Spatial frequency domain imaging (SFDI), FDPM is developed to quantitatively

measure optical parameters in turbid tissue. An intensity-modulated laser pulse is projected into the tissue to analyze the optical properties. Moreover, the measured frequency-dependent diffusion with different source-detector distances is compared with the theoretical diffusion model. The fitting of the diffusion equation with measured data provides the values of optical parameters such as absorption and reduced scattering. It has been found that the probing depth of this technique varies with the RF carrier frequency. However, its spatial resolution is relatively poor due to multiple light scattering. In a turbid sample, the multiple scattering effects broaden the laser pulse spatially and temporally. When combined with the time-gating technique, FDPM can measure the time-dependent light propagation using a time-gating detector to select the viewing location. Therefore, temporal selection can confine the measured signal from a specific region in the tissue.

- *Adaptive Optics (AO) / Wavefront Shaping (WS)*

Adaptive optics (AO) [32, 33] is a promising method to correct wavefront aberration from the target behind a highly scattering medium. The distorted image can be improved and recovered by the AO correction, and this technique can be integrated with standard optical methods such as microscopy [34], including confocal microscopy [35] and two-photon microscopy [36, 37]. The wavefront aberration could come from the imperfection of the optical system, the refractive index mismatch, and the inhomogeneous structure of biological samples. However, adaptive optics mainly work with the paraxial waves in the region where the scattering does not dominate light propagation [38]. Therefore, despite the successful aberration correction in biological samples, highly multiple

scattering is still another difficulty for adaptive optics. In the deep layer of a highly scattered medium, the AO loses its ability to reconstruct the image since most information has been lost.

Wavefront shaping [38-44], different from AO, is designed to generate maximum constructive interference at a small point behind a highly scattering medium. Instead of using a distorted image for wavefront correction in adaptive optics, wavefront shaping methods use scattered light for incident light modulation. Due to the capability of controlling the wavefront of scattered light, wavefront shaping is a promising technique for enhancing and maximizing the signal at a specific point inside or behind a scattering medium by significantly increasing the contrast from the background. The significant difference between WS and AO is that WS mainly works for samples with high scattering coefficients where AO cannot reconstruct imaging from entirely scattered light in such samples. The technique works for 2D imaging through a highly disordered medium using linear [45] or nonlinear optical techniques [46, 47]. In this study, we choose wavefront shaping as the primary technique to improve depth-sensitive spectroscopy. In **Chapter 2**, we introduce the development of the current wavefront shaping techniques.

1.2 Objectives

In this dissertation, our overall objective is to develop feedback-based wavefront shaping to improve optical measurements' depth sensitivity. In the course of the Ph.D. research, the following specific aims are progressively achieved as described below.

1.2.1 Aim 1: To Improve the Depth Sensitivity of Measured Optical Spectra with Feedback-based Wavefront Shaping.

The digital conjugation method [48-50] and transmission matrix measurements [51, 52] are prevalent in wavefront shaping. While the techniques involving the direct measurements of wavefronts are relatively fast, they typically only consider the enhancement effect of phase changes, thus only working well for enhancing coherent light, such as laser light. This limitation induces two disadvantages. One is that this approach does not fully take advantage of the power of wavefront shaping when used for incoherent light enhancement, such as in fluorescence or Raman measurements. The reason for this disadvantage is that the direct measurement approach only optimizes the excitation part, i.e., laser light propagation from the light source to the target buried under the surface of a sample, of the fluorescence or Raman detection without considering the emission part, which refers to the fluorescence or Raman light propagation from the target to the optical detector. The other disadvantage is that this approach would not work well for samples in which a target differs from the surrounding region, primarily in fluorescence or Raman properties. This can happen in many applications, such as cancer diagnosis with external fluorescence or surface-enhanced Raman scattering

(SERS) contrast agents, where the contrast agent is more concentrated in the tumor region due to the enhanced permeability and retention (EPR) effect or the mechanism of active targeting. In this case, there is no prominent guide star at the laser wavelength, but there is in the fluorescence/Raman band. Feedback-based wavefront shaping is more versatile than other wavefront shaping methods for particular conditions because it can work for both coherent and incoherent light by selecting an appropriate light wavelength for generating the feedback signal.

The performance of feedback-based wavefront shaping is strongly affected by the quality of feedback signals. To increase the enhancement and refocusing capability, isolation of the target signal from the background is essential for optimization. Using both coherent and incoherent feedback makes the approach available for fluorescence and Raman scattering. We proposed some methods to increase the signal-to-noise ratio of the target signal to improve each iterative optimization, which is not only for laser speckle but also for incoherent signal enhancement.

1.2.2 Aim 2: To Achieve Real-Time Feedback-Based Wavefront Shaping for Dynamic Samples

In the literature, feedback-based wavefront shaping is conducted in 2D using a micro-electromechanical systems spatial light modulator (MEMS-SLM), whose highest refreshing rate of the device for phase modulation is around 10 kHz currently [53]. Instead of liquid crystal used in LCOS-SLM, MEMS-SLM uses deformable micromirrors to adjust the optical path length of incident light with the piston movement of these micromirrors. The high-speed actuator motion allows it to change the incident wavefront much faster than liquid crystal. With

measurements and data processing during the optimization, the actual phase modulation rate can reach approximately 4.1 kHz for each degree of freedom. In feedback-based wavefront shaping, the refreshing rate of wavefront optimization in terms of the number of optimization rounds completed per unit of time depends on the degrees of freedom and the iteration times required in each round of optimization in addition to the phase modulation rate of the device. A more significant number of degrees of freedom leads to a higher enhancement factor, a long time for each iteration, and a lower refreshing optimization rate. We propose to speed up feedback-based wavefront shaping further using an acousto-optic deflector. Compared to 2D wavefront optimization using SLM or DMD, AOD can only optimize the wavefront in 1D but with a significant advantage in the refreshing rate. The time delay between the initiation of the driving RF and the deflection of the entire incident light due to the limited velocity of ultrasound and finite beam diameter ultimately imposes an upper bound on the refreshing rate of a dynamic event that can be tracked instantly by the device from a few hundred kilohertz to a couple of megahertz. In other words, we can control the refreshing rate of an AOD, which needs to modulate the entire beam, using a laser beam with a different diameter. For instance, the acoustic speed in the crystal of the AOD used in our setup is 0.66 mm/ μ s. Therefore, it is possible to get a 1-MHz refreshing rate of phase modulation using a laser beam with a size of 660 μ m. The rate is already much faster than most SLM/DMD-based techniques, in which the highest refreshing rate of the device is 350 kHz using 1D MEMs-SLM [54]. We aim to demonstrate the power of feedback-based wavefront shaping for improving the depth sensitivity of spectroscopic

measurements to a target using an AOD in a scattering tissue phantom, a fluorescent tissue phantom with strong scattering, and finally, in actual tissues.

1.2.3 Aim 3. To Accelerate Depth-Sensitive Optical Measurements

Compared to a point scanning confocal microscopy, line-shape excitation has the benefit of high-speed fluorescence imaging by avoiding one-dimensional scanning. However, such techniques only perform lateral scanning, while depth-sensitive optical measurements require axial scanning. In contrast, the simultaneous measurements of signals from all depths will enable quick depth-sensitive optical measurements. We propose using AOD to generate a series of radiofrequency-coded light beams, each beam with a different radiofrequency focused on a different depth in a sample. Due to the difference in coding radiofrequency, the sum of signals from all depths can be recorded by a single-pixel detector with high bandwidth. The signal from each depth can be reconstructed in the frequency domain by taking Fourier Transform (FT). This study's target depth range is around 0 to 200 μm , suitable for epidermal measurements in rat skin with at least ten resolvable spots along the depth.

1.3 Outline of Dissertation

The dissertation describes the principle and the application of signal enhancement by wavefront shaping in a disordered medium. The optical setup was designed individually for each experiment in every chapter. Overall, the dissertation is organized into eight chapters, as summarized below:

In **Chapter 1**, we introduce the common background and general motivation for the central theme of the dissertation, i.e., to overcome the influence of multiple light scattering in depth-sensitive optical spectroscopy. The outline of the dissertation is provided at the end of the chapter.

Chapter 2 introduces the specific background in wavefront shaping techniques developed to solve the multiple light scattering issue. Various types of wavefront shaping, their application in biomedical optics, and prior knowledge about feedback-based wavefront shaping for depth-sensitive spectroscopy are summarized to brief those readers unfamiliar with the topics. Note that each study in this dissertation has its introduction to motivate the particular research.

Chapter 3 presents the first study regarding improving wavefront shaping by a spatial light modulator with spatial and spectral filtering techniques. This method can increase the signal-to-noise ratio by isolating the target signal from the disordered media, effectively improving the performance of feedback-based wavefront shaping.

In **Chapter 4**, we report a method of speeding up wavefront shaping with AOD modulation, which differs from SLM modulation. In addition to the evaluation result of this technique, its potential application is also discussed.

In **Chapter 5**, we present the improvement of weak signal enhancement with lock-in detection using AOD, which is the continuation of the study in the previous chapter. The combination of the lock-in method and AOD modulation significantly improves the signal-to-noise ratio to enhance the effectiveness of the feedback-based wavefront shaping in case of weak light such as fluorescence or Raman light. The proposed method thus carries great potential in applications involving weak light.

Chapter 6 describes our effort in accelerating wavefront shaping with FPGA to make it possible to apply to dynamic samples with a short correlation time. The proposed method can potentially perform feedback-based wavefront shaping during *in vivo* measurements with a reflected optical setup.

Chapter 7 reports the final study regarding speeding up depth-sensitive optical measurements by measuring all signals in a snapshot. This technique increases the speed of depth-sensitive optical measurements by encoding the information from each depth to a unique radiofrequency. It improves the SNR by taking AC measurements in the RF range to reduce $1/f$ noise.

Chapter 8 summarizes all the studies and contributions of this dissertation and provides the outlook and prospect of our techniques in biomedical optics.

Chapter 2

Wavefront Shaping

Unlike other techniques for overcoming multiple scattering, wavefront shaping turns scattered light into valuable signals. Unlike absorption, scattered light maintains its original frequency and energy during propagation. The consistency of light properties makes it possible to be utilized rather than rejected in detection. Early articles published in 1990 successfully showed that light scattering caused by disordered media carries information that can be retrievable with the memory effect [55]. Vellekoop and Mosk [56, 57] used and implemented the concept in 2007. They refocused the light through a highly scattering medium by modulating the phase of an incident light beam. It provides a crucial breakthrough in applying the technique in the visible region, potentially more helpful for biological application. It is noted that visible light used for wavefront shaping offers a higher spatial resolution due to its shorter wavelength than other sources, such as near-infrared light, which is less affected by light scattering in tissue but offers a lower resolution. The higher energy requirements for light at a longer wavelength to achieve reasonable excitation efficiency, e.g., to excite fluorescence, is another potential issue when selecting the light source. Hence, light with a shorter wavelength with higher energy for excitation in small areas has advantages in biomedical imaging. The potential applications of focusing light into a scattering medium include but are not limited to fluorescence or

Raman spectroscopy, deep imaging microscopy, optical tweezer, and multimode optical fiber [58]. Wavefront shaping can potentially improve and broaden the ability to exist optical techniques for better performance. In this chapter, we discuss the principle, representative applications of wavefront shaping, and particularly feedback-based wavefront shaping in detail.

2.1 Principle of Wavefront Shaping

Since scattering is dominant over absorption in most biological samples [11], it is possible to exploit substantial degrees of freedom to control scattered light in a scattering medium. Elastic light scattering in a stationary scattering medium is deterministic instead of random, so the information in scattered light can be recovered in theory. Therefore, instead of removing scattered light, wavefront shaping utilizes scattered light to achieve refocusing. Due to the deterministic nature of scattering, the scattering pattern in a static sample is fixed and linearly correlated with the incident light field. In diffusion theory, the transmission matrix can describe the linear relationship as shown in **equation (2.1)** [59].

$$E_b = \sum_a^N t_{ba} E_a \quad Eq. (2.1)$$

E_a and E_b indicate the incident and transmitted light field, respectively. t_{ba} represents the transmission matrix. The labels (a, b) are the free modes of the input (incident light) and output (transmitted light), respectively. N is the total number of input-free modes. The intensity of the transmitted light can be represented as **equation (2.2)**:

$$I_b = |E_b|^2 = \frac{|\sum_a^N t_{ba} E_a|^2}{\sqrt{N}} \quad Eq. (2.2)$$

Wavefront shaping is to find the best solution that maximizes the output normalized light intensity (I_b) on a specific spatial location by manipulating the input light field (E_a) with fixed t_{ba} . Hence, with a specific incident field (E_a), light can form highly constructive interference at a target point and enhance the incident energy on the target. It has been experimentally proved that scattered light can be focused on a target inside or after a turbid medium with wavefront shaping by manipulating the phase and amplitude of incident light [40, 56]. However, since the degrees of freedom is usually much smaller than the number of actual scattering modes, most light still does not contribute to the focusing area even after wavefront shaping. Even so, the increased contrast of the target from the background has made it potentially very useful in some applications. The enhance factor ($\eta = \frac{I^*}{\langle I_0 \rangle}$) is commonly used to quantify enhancement from wavefront shaping, which is defined as the ratio of the intensity at the focal spot (I^*) to the average background (I_0). The theoretical enhancement factor can be calculated from **equation (2.3)** [38]:

$$\eta = \alpha(N - 1) + 1 \quad \text{Eq. (2.3)}$$

where N is the degrees of freedom, α is the modulation factor. For phase-only modulation, $\alpha = \frac{\pi}{4}$, and for phase and amplitude modulation together $\alpha = 1$. Wavefront shaping aims to find the optimal phase and amplitude distribution of incident light to form constructive interference on the target. With modulated incident light, the effect of multiple scattering can be compensated. Hence, wavefront shaping provides an alternative way for imaging through a turbid medium and focusing light in turbid media using linear [45] or nonlinear optical

techniques [46, 47]. Moreover, thanks to the scattering property, it can break the diffraction limit imposed by the numerical aperture (NA) of the objective and creates a focal spot beyond the diffraction limit [41, 60] as an “opaque lens” [61]. Beam manipulation can recover lost information due to light scattering. Because every sample has a different transmission matrix, an optimized wavefront must be obtained individually. The specificity of the relationship between each sample’s transmission matrix (often unknown) and optimized wavefront requires a universal way to determine the optimized wavefront. In general, there are three standard wavefront shaping techniques to modulate the particular incident field (E_a) for compensating the multiple scattering effects, i.e., transmission matrix (TM) measurements, optical phase conjugation (OPC), and feedback-based wavefront shaping. The following sections will introduce the principle of each technique.

2.2 Three Types of Wavefront Shaping Techniques

2.3.1 Measurements of the Transmission Matrix (TM)

- *Measurements of Transmission Matrix (TM) with Spatial Light Modulator (SLM)*

TM measurement method directly gets the information from the transmission matrix (t_{ba}) and use it to modulate the input channel [43, 51]. The transmission matrix (TM) measurement retrieves information about the relation between input and output light for the scattering matrix. SLM is a versatile device used to measure the transmission matrix [51, 52]. With the TM, focusing light through a scattering sample can be conducted by modulating all the input light channels corresponding to one specific output channel. By using this, scattered light's spectral and polarization properties can also be recovered by analyzing the measured transmission matrix [62]. In addition to coherent laser light [56], the transmission matrix can also be measured for fluorescence or photoacoustic signals [51]. Another application of the technique is using a multispectral light source for spatiotemporal focusing [63]. The multispectral transmission matrix of the light source provides the ability to control light in a scattering medium spectrally. However, multiple measurements are required to get the transmission matrix of a scattering medium, and more optically controllable degrees of freedom need more measurements. The light modulator's refreshing rate and data analysis limit TM measurements' speed. The measurement of the full scattering matrix of a turbid sample is highly time-consuming. Fortunately, in most applications, only part of the scattering matrix is required for optimization.

Another concern is that the correlation between input and output channels is valid for reuse only when the memory effect is present. In a scattering medium, the memory effect exists within a limited range of sample thickness, which is proportional to $\sqrt{\frac{\ell_{tr}}{L}}$ (ℓ_{tr} : transport mean free path; L : thickness) [64].

- *TM measurements with Digital Micromirror Devices (DMD)*

Even though an SLM has high pixel resolutions, its low refreshing rate is a severe limitation. Compared to the SLM, a digital micromirror device (DMD) has a relatively higher refreshing rate of up to 23 kHz [65], so wavefront optimization is much faster. However, unlike the ability to perform phase modulation with SLM, DMD modulates the binary amplitude of a wavefront, limiting enhancement performance. Despite the limitation, it has been shown that the binary amplitude modulation can successfully refocus light in scattering media with a high enhancement factor [65, 66], and the performance of DMD-based modulation is comparable to LCoS-SLM [67]. Another technique developed recently is parallel optimization using the Hadamard transform implemented by a high-speed DMD with large degrees of freedom. It is reported that the enhancement factor can be greater than 100,000 by exploiting the megapixels on a DMD in around an hour [68]. In the exact condition of using megapixels, 19 hours is required for enhancement by using SLM.

- *TM Measurements with Microelectromechanical Systems Spatial Light Modulator (MEMS-SLM)*

Compared to LCoS-SLM (60 Hz), the MEMS-SLM can update the phase at a much higher refreshing rate (> 10 kHz). The higher refreshing rate of the MEMS-SLM makes fast optimization possible for dynamic samples. With the integration of the high transfer rate of a field-programmable gate array (FPGA), the optimization time can drop down to somewhere between tens of microseconds and sub-millisecond [53]. So far, the fastest MEMS-SLM, to our knowledge, is made by a grating light valve (GLV). The high-speed 1D MEMs-SLM made of GLV can operate at a rate of up to 350 kHz and complete the phase correction of wavefront shaping in a few milliseconds [54]. However, the pixel number (~ 1000 pixels) in the MEMS-SLM is much smaller than the LC-SLM (1920×1080 pixels), and so are the degrees of freedom that can be modulated.

2.3.2 Optical Phase Conjugation (OPC)

The optical phase conjugation (OPC) approach measures the speckle field originating from the target source and uses the information to focus light in the opposite direction [13]. Since the optical information from a source passing through a scattering matrix is entirely reversible, the generation of another light beam in the reverse direction with the conjugated phase of the speckle field will propagate through the scattering matrix with the same optical path and focus light on the source.

- *Digital Optical Phase Conjugation (DOPC)*

Digital optical phase conjugation (DOPC) becomes a promising method with a microsecond-scale focusing rate suitable for *in vivo* applications by eliminating the iterative process [48-50]. The DOPC optimizes the signal by recording the scattered field of one light beam through a scattering sample when it starts propagation from a target spot and then initiates another light beam with the conjugate scattered field from the other side of the medium in the opposite direction. Due to the reciprocity of light propagation, the second light with the conjugate scattered field will travel back to the target spot by following the same optical path. According to the principle, some advanced techniques based on optical phase conjugation (OPC) and ultrasonic guide star are also developed for biological applications, such as time-reversed ultrasonically encoded light (TRUE) [69, 70], time reversal of variance-encoded light (TROVE) [71], and time-reversed ultrasound microbubble encoded (TRUME) [72]. The time-reversal compensation for overcoming the scattering effect also has been used to improve OCT [73] for deep tissue imaging. Besides, the snapshot measurement for wavefront shaping allows high-speed focusing that can be applied to dynamic samples [74].

- *Optical Phase Conjugation with Acoustic-Optical Deflector (AOD)*

When the acoustic-optical deflector (AOD) is combined with the digital phase conjugation method and pulse laser source, the change of phase on the acoustic wavefront of different frequency components can rapidly modulate the wavefront of the laser beam on a microsecond scale to complete the whole procedure [75].

The frequency-encoded spatiotemporal (FEST) [76] focusing technique is another method for high-speed wavefront shaping for *in vivo* applications. FEST combines the AOD and SLM for direct light modulation. In this principle, each laser beam carrying a specific frequency from the AOD is projected on SLM. By analyzing the frequency components in the target signal, the phase information of each signal with different frequencies can be measured. With the phase information, the digital phase conjugation method can be used to optimize the incident light for canceling the scattering effect.

2.3.3 Feedback-Based Wavefront Shaping

Unlike TM measurement and OPC techniques, feedback-based wavefront shaping iteratively searches the optimized input matrix to get the desired output without measuring the wavefront. Without being limited by the memory effect, i.e., within a certain depth that the reversibility of the wave equation is still applicable, feedback-based wavefront shaping optimizes the phase and amplitude of incident light based on the feedback signal from a sample [38, 77]. The source of the feedback signal could be coherent laser light, fluorescence [39, 45, 78], Raman scattering [79], non-linear [36], and photoacoustic signal [80]. The time-gating [20, 22] signal from singly scattered light can also serve as a feedback signal for optimization. However, the time-gating method has only been demonstrated for reflectance measurements, likely due to the weak signal. The optimized wavefront shaping from the feedback wavefront shaping is similar and close to optical phase conjugation.

For this wavefront shaping technique, the requirement of a guide star limits its applications because the intrinsic guide star may not be available.

Meanwhile, an external guide star [81] is inaccessible in some applications, for example, clinical scenarios. Although an internal guide star utilizing nonlinear optical focusing or internal absorption contrast in tissues is beneficial [36, 82], the appropriate methods reported in the literature either involve a complicated setup or are not applicable in depth-sensitive optical spectroscopy. Even though these techniques can increase the spatial resolution and the penetration depth, losing the most scattered light with an increasing depth strongly decreases the signal-to-noise ratio and lead to challenges in applications on thick biological tissues.

2.3 Application of Wavefront Shaping in Biomedical Optics

2.3.1 Optogenetics and Optical Tweezer

Optogenetic stimulation [83, 84] is an essential technique to directly stimulate the neurons to trigger the action of light-activated enzymes or ion channels in neuroscience. However, even with soft tissue like the brain tissue, multiple light scattering in the tissue significantly reduces the temporal and spatial resolution that hinders single neuron activation. High spatiotemporal foci formed by wavefront shaping can be used more precisely for optogenetic manipulation of brain neurons to overcome this issue. The time-reversed ultrasonically encoded light (TRUE) method [15] and parallel wavefront optimization method [85, 86] have been proposed for optogenetic stimulation on mouse brain slices. Another example is to perform beam shaping with generalized phase contrast (GPC) [87] or computer-generated holography (CGH) to generate multiple focus spots. The

technique has been used to stimulate various neuron cells of drosophila larvae [88]. Besides the stimulation, the precise focusing energy can be used to control microparticles and nanoparticles as optical tweezers. With wavefront shaping, the light can be focused on the area with a size below the diffraction limit to manipulate particles in disordered media [89, 90].

2.3.2 In vivo Deep Tissue Imaging

One of the essential applications of wavefront shaping is the *in vivo* measurement of live animals. Spectral measurements or 3D imaging from certain tissue areas provide helpful information for diagnosis without dissection. Compared to *ex vivo* and *in vitro* environments, *in vivo* measurement yields more helpful information for diagnosis. Wavefront shaping has been used to increase the penetration depth and spatial resolution for label-free optical measurements *in vivo* in techniques such as two-photon microscopy [91] and optical coherent tomography (OCT) [73, 92-94]. Due to the memory effect, biological imaging [95] can be conducted in a large area from a scattering tissue with the same optimized wavefront of the incident light with tilted scanning [64]. Wavefront shaping for achieving subcellular resolution in tissues has been used for brain imaging [96, 97] and the study of eye cataracts [98].

2.4 Feedback-Based Wavefront Shaping for Depth-Sensitive Spectroscopy

There are several benefits when using feedback-based wavefront shaping in depth-sensitive spectroscopy. First, the linear relationship between the output speckle field and incident light field is effective in a thin scattering sample. When the thickness increases and so does the number of scattering events, the DPOC [48, 49, 99] has only demonstrated with acoustic guiding that the long wavelength of the acoustic wave limits the resolution. Feedback-based wavefront shaping [38, 100], on the other hand, can work on visible light for high resolution. Second, the method works for both coherent and incoherent light in a transmission or reflection setup. The versatile choices of feedback enable the technique to be applied to different measurements in which DOPC is unable to work, such as fluorescence measurements under two-photon excitation. Third, the simplicity of this technique makes it easier to develop. More detailed information about feedback-based wavefront shaping related to this study is shown below.

2.4.1 *Feedback Signal*

The concept of feedback-based wavefront shaping maximizes the target signal by adjusting the phase and amplitude of the input light and watching the trend in a feedback signal that is correlated to the target signal. Finding a guide star [77], which is precisely the target or close to the target with known properties, is desirable to ensure the correlation between the feedback signal and the target signal. The feedback signal should be originated from the guide star. Because the quality of the feedback signal determines the performance of wavefront shaping,

the choice of the guide star is crucial. The laser speckle at the desired position measured with a photodetector can be used as the feedback signal in the laser refocusing through a scattering medium. The iterative optimization can directly increase the intensity of the feedback signal and generate a high contrast relative to the background by increasing constructive interference. While phase modulation is only applied to laser light, fluorescence emission can be used as the feedback signal to improve the focusing of laser light on a fluorescent sample through a scattering medium. Similarly, Raman light can also be used as the feedback signal when the aim is to maximize Raman signals.

2.4.2 Optimization Algorithm

The goal of wavefront shaping is to maximize the signal from the target region. To find the “global maximum,” some mathematical methods are required. Simulated annealing (SA) [101], genetic algorithm (GA) [102], partitioning algorithms (PA) [103], continuous sequence algorithm (CSA) [103], and optimal steepest ascent (OSA) [104] algorithm have been reported in the literature. In this section, we introduce the two common ones used in the study: CSA and OSA.

- *Continuous Sequential Algorithm (CSA)*

Several algorithms can be used in feedback-based wavefront shaping to maximize localized intensities iteratively. The first one used in the research is the continuous sequential algorithm (CSA) [103]. The principle of the method is illustrated in **Fig. 2.1**. In the continuous sequential algorithm, one will find the optimal phase of each grid, corresponding to one degree of freedom, on a spatial light modulator by scanning in the range from 0 to 2π with a given increment

(0.2π) . The phase with maximum intensity will be chosen as the final value. This iterative scanning process is applied to each grid sequentially. In other words, each pixel of SLM will optimize its phase after the previous one is determined. The optimization process is completed when the phase of the last grid is determined. Despite the advantage of its straightforward principle, the fixed phase step limits the performance of the phase optimization. Another disadvantage is that the method likely leads to the local maximum of a target signal instead of the global maximum compared to other methods below due to the neglect of many possible phase distributions.

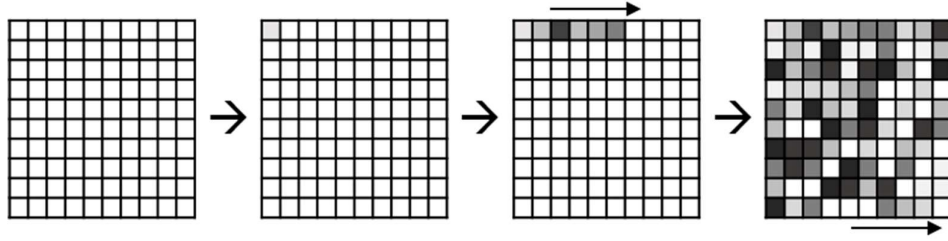


Fig. 2.1. Illustration of the continuous sequential algorithm (CSA).

- *Optimal Steepest Ascent (OSA) Algorithm*

The second method is called the Optimal Steepest Ascent (OSA) [104]. The principle is illustrated in **Fig. 2.2**. For convenience, a phase vector is defined as the 1-D tabulation of phases at all degrees of freedom, i.e., all pixels on the SLM or all RFs for the AOD, on the phase modulator (SLM or AOD). The following equation can express the iterative optimization process.

$$S_{i+1} = S_i + \mu \cdot \nabla C \quad \text{Eq. (3.1)}$$

where S_{i+1} represents the optimized phase vector after the $(i+1)$ -th iteration, S_i is the phase vector after the i -th iteration, μ is the phase step size, and ∇C is the normalized gradient of the feedback signal for the phase vector. The feedback signal could be light intensity or amplitude. The gradient defines the contribution of each element in the phase vector that corresponds to each pixel on the SLM or each RF component in the AOD on the feedback signal. Since all the elements in the phase vector contribute to the feedback signal, partial derivatives must be measured sequentially, in which one element is varied every time. Once the gradient value is determined, we can use the step size μ to calculate the optimized phase corresponding to the signal with the maximal contribution from the target. Multiple iterations may be required until the result converges. Compared to CSA, OSA spends less time with fewer steps to achieve maximum intensity. Unlike the CSA, which calculated the phase of each grid sequentially and individually, OSA determines the new phases of all grids simultaneously. Therefore, OSA requires much less time to reach the local optimum than CSA.

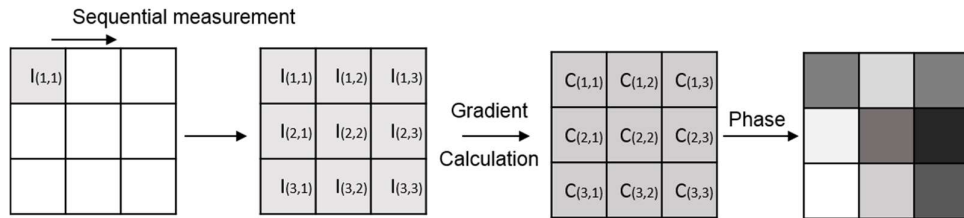


Fig. 2.2. Illustration of the Optimal Steepest Ascent (OSA) algorithm. Each grid represents one element of the phase vector. In each step, $I_{(i,j)}$, which represents the partial derivative at the pixel (i, j) in the SLM, is measured for every element of the phase vector sequentially. After all the partial derivatives are measured, the normalized gradient of every element can be calculated. In the final step, the optimized phase can be calculated using Eq. (3.1) from the normalized gradient.

2.4.3 Limitations of Feedback-Based Wavefront Shaping

- *Long Iteration Time*

Compared to the transmission matrix measurement method or optical phase conjugation requiring one or a few measurements, the iterative loop in feedback-based wavefront optimization takes much longer to reach the maximum intensity. The requirement of this time-consuming process hinders the possibility of achieving high-speed wavefront shaping, thus not suitable for dynamic samples. To overcome the issue, decreasing the time for each iteration or the number of required iterations can potentially measure a sample with a short correlation time.

- *Signal-to-Noise Ratio (SNR)*

Essentially, the performance of feedback-based wavefront shaping is strongly affected by the SNR of the feedback signal. Iterative optimization can lead to a strong signal (constructive interference) only if it is based on a stable feedback signal with a decent SNR.

2.4.4 Improvement of Feedback-Based Wavefront

Shaping

In **Chapters 3 to 6**, we proposed multiple methods to improve feedback-based wavefront shaping regarding SNR and speed. By increasing the contribution of the target to the feedback signal with spatial and spectral filtering (**Chapter 3**) and the SNR of the feedback signal using lock-in detection (**Chapter 4 and Chapter 5**), significant improvement in optimization can be achieved in a disordered sample with high scattering. Then by combining AOD modulation and FPGA acceleration (**Chapter 6**), the speed of wavefront shaping can be increased dramatically to catch up with dynamic samples with a short correlation time.

Chapter 3

Improving Depth-Sensitive Fluorescence Spectroscopy with Wavefront Shaping by Spectral and Spatial Filtering

3.1. Introduction

Feedback-based wavefront shaping is known for its versatility [38, 77]. However, one well-known weakness of this technique is the requirement of a guide star. An external guide star [81] is inconvenient or sometimes even impossible in future clinical applications. Although an internal guide star utilizing nonlinear optical focusing or internal absorption contrast in tissues is beneficial [36, 82], the relevant methods reported in the literature either involve a complicated setup or are not applicable in depth-sensitive optical spectroscopy. Without these special guide stars, the past study for depth-sensitive Raman spectroscopy has shown only slight enhancement [79], which suggests that the enhancement for fluorescence spectroscopy would be even poorer due to potential photobleaching. To address this problem, we propose to explore two standard optical techniques that have not been fully exploited in feedback-based wavefront shaping to our best knowledge, i.e., spatial filtering and spectral filtering, in an attempt to increase the depth sensitivity to a subsurface target in depth-sensitive fluorescence spectroscopy. The advantage of using fluorescence as the feedback signal is that it is suitable for any target with low scattering contrast. In such a case, it is challenging to use reflected

excitation light as the feedback signal for light optimization since it may be too weak to detect. Moreover, the depth-dependent distribution of endogenous fluorophores in some tissues, such as epithelial tissues, could be utilized to increase the depth sensitivity to a particular layer. Our results show that the depth sensitivity to fluorophores in the bottom layer of a two-layered tissue phantom in a reflection setup can be enhanced significantly with proper spatial filtering and spectral filtering of guiding light for wavefront optimization compared to the situation without such filtering. The filtering is similar to coherence or time gating, i.e., selecting the light from the target zone for wavefront shaping.

The current gap in this area is that the past study in depth-sensitive optical spectroscopy using wavefront shaping without special guide stars showed only slight enhancement (around ~20% increase) [79], which is insufficient to achieve any meaningful result. Our main contribution to this study is to demonstrate that the enhancement factor using wavefront shaping for depth-sensitive optical spectroscopy can be increased to about 300% with the help of spatial filtering and spectral filtering. This has not been reported previously for depth-sensitive optical spectroscopy in feedback-based wavefront shaping. Therefore, our work represents a new advance toward applying wavefront shaping in depth-resolved optical spectroscopy. Moreover, we show that a trade-off needs to be made on the filtering parameters because of the positive effects of spatial/spectral filtering in wavefront shaping performance and the decreasing signal-to-noise ratio associated with filtering.

3.2. Principle and Methodology

3.2.1. Experimental Setup

The schematic of the optical setup is shown in **Fig. 3.1**. Continuous-wave coherent laser light (Millennia eV 5W, Spectra-Physics, Santa Clara, California, USA) is cleared up by passing through a spatial filter, which is then projected onto a Spatial Light Modulator (SLM, PLUTO-2-VIS, HOLOEYE, Berlin, Germany) with a resolution of 1920 x 1080 pixels. A half-wave plate and a linear polarizer are used to adjust the power and polarization to match the requirement of the SLM. The light is expanded to fill the SLM as much as possible for optimal modulation. The modulated light is demagnified first and then focused onto the sample by a 50x microscope objective (UMPlan FI; Olympus, Shinjuku, Tokyo, Japan) with an NA of 0.8 and a working distance (WD) of 0.6 mm. The same microscope objective collects the excited fluorescence light excited from the sample. The sample is mounted on a three-dimensional stage (PT3/M; Thorlabs, Newton, New Jersey, USA) for precise adjustment. A long-pass dichroic mirror separates the emitted fluorescence from the excitation light (DM; Di03-R532; Semrock, Rochester, New York, USA). A long-pass filter (BLP01-532R-25; Semrock, Rochester, New York, USA) afterward further isolates the fluorescent light. For evaluating spectral filtering on wavefront shaping, none or one of the two bandpass filters (FF01-618/50; FF01-650/60; Semrock, Rochester, New York, USA) is placed right behind the long-pass filter to select the desired spectral band of fluorescence.

A photomultiplier tube (PMT, 77348, Newport Corporation, California, USA) driven by a high voltage power supply (70705, Spectra-physics, Santa Clara, California, USA) and preceded by a preamplifier (SR560; Stanford research systems, Sunnyvale, CA, USA) is used to detect fluorescence for SLM

optimization. When the effect of spatial filtering is evaluated, several pinholes with a range of diameters (P800H, P200H, P150H, P100H, and P75H, Thorlabs, Newton, New Jersey, USA) are placed on the image plane one at a time to prevent unwanted light from being captured by the PMT or spectrometer. Once the optimization is complete, the spectrum will be recorded by a PI spectrometer that consists of a spectrograph (Acton SP2150; Princeton Instruments, Trenton, New Jersey, USA) and a CCD camera (PIXIS 400; Princeton Instruments, Trenton, New Jersey, USA). To increase light collection efficiency after the pinhole, a condenser with a magnification of 0.5 is placed before a round-to-linear fiber bundle (BFL105HS02, Thorlabs, Newton, New Jersey, USA) with an effective core diameter of 355 μm on the round end, and the size of the linear end matches the input slit width of the spectrometer.

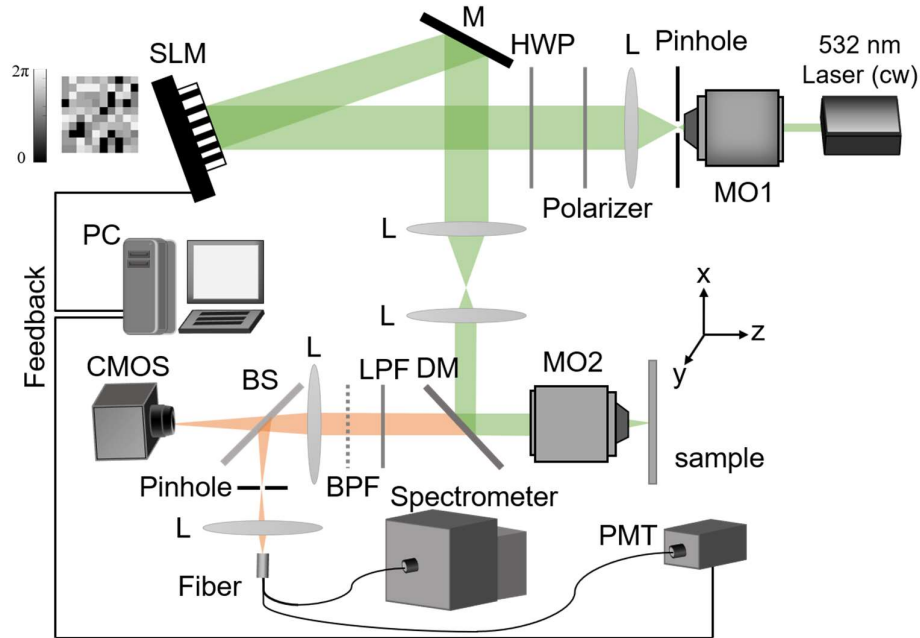


Fig. 3.1. Experimental setup. M: Mirror; L: lens; HWP: half-wave plates; LPF: 532 nm long-pass filter; BPF: band-pass filter; SLM: spatial light modulator; MO1: 20 $\times$ microscope objective; MO2: 50 $\times$ microscope objective; BS: beam splitter; DM: 532 nm dichroic mirror; PMT: photomultiplier tube.

3.2.2. Phantom Preparation

Scattering tissue phantoms were made by mixing titanium dioxide (TiO₂ with 1.5 μm particle size; US Research Nanomaterials Inc., Houston, USA) in dissolved polydimethylsiloxane (PDMS, Sylgard 184; Dow Corning, Midland, Michigan, USA) [105]. The reduced scattering coefficient (μ_s') was adjusted by varying the concentration of TiO₂ in PDMS according to the calculation using Mie theory with known refractive index, particle size, and volume fraction [106]. Purple fluorescent microparticles (FH-2062-2; Spherotech, Libertyville, IL, USA) and Rhodamine 6G (R6G 56226; Sigma-Aldrich, St. Louis, MO, USA) were separately suspended in isopropyl alcohol (IPA W292907; Sigma-Aldrich, St. Louis, MO, USA) first, then were added into different TiO₂ phantoms in PDMS before setting. Every sample was continuously stirred on a magnetic stirring hot plate for at least 1 hour to ensure the homogeneous distribution of particles or R6G in PDMS. The concentration of the particles was adjusted to around 3×10^7 particles/g in PDMS, and the concentration of R6G was 128 μM in PDMS. Then each sample was mixed with a curing agent so that PDMS was set to form a solid phantom. A two-layered sample was made by stacking one layer with R6G on top of the other with the fluorescence microparticles. The top layer's transport mean free path ($l_t = 1/\mu_s'$) was around 3.3 mm with a thickness of $110 \pm 10 \mu\text{m}$ to mimic the epithelium in epithelial tissue [107]. The transport mean free path of the bottom layer was around 10 mm with a thickness from $110 \pm 10 \mu\text{m}$ to serve as the superficial region of the stroma.

3.2.3. SLM Modulation

The continuous sequential algorithm [103] was used to update the phase values of the SLM in the study. A total of $1,000 \times 1,000$ pixels on the SLM were binned into 10×10 grids, i.e., 100×100 pixels in each grid. The phase shift from 0 to 2π with an increment of 0.2π was utilized during optimization with a total of 1,000 iterations in each experiment. The algorithm aims to optimize the feedback signal by individually modulating the phase for each grid in the spatial light modulator. In the iteration process, the feedback signal will be measured with a different phase for each grid in a range of 0 to 2π . After the measurements, the phase of the segment will be set to the value that yields the maximum feedback signal. The process will continue for each grid until the phase values of all grids are set. Due to its high sensitivity, a PMT was used to collect fluorescence during wavefront optimization. The PMT reading served as a feedback signal in each iteration for wavefront optimization so that it would be maximized at the end of wavefront shaping. After the optimized wavefront, the fluorescence spectrum was measured using a commercial spectrometer.

3.2.4. Enhancement Factor and Emission Ratio

The fluorescence spectra of pure R6G and purple fluorescent particles at low concentrations are shown in **Fig. 3.2(B)**. The spectral contribution from each fluorophore is estimated by curve fitting, in which a spectrum measured from a two-layered sample, as shown in **Fig. 3.2(A)**, is modeled as the summation of the spectral contributions from pure R6G and fluorescent particles, each linearly related to the respective concentration. The spectrum contributed by each

fluorophore is integrated from 540 nm to 670 nm to estimate the fluorescence contribution for calculating the enhancement factor and emission ratio.

The enhancement factor is defined as the ratio of fluorescence contribution from the purple fluorescence particles after wavefront optimization to that before optimization [53], both after background subtraction.

For a two-layered sample with a different fluorophore in each layer, the emission ratio, defined as the ratio of fluorescence contribution from purple fluorescent particles to the fluorescence contribution from R6G, is used to quantify the sensitivity of the collected signal to the bottom layer.

3.2.5. Spectral and Spatial Filtering

The effect of spatial filtering on the performance of wavefront optimization is evaluated for a series of pinholes with a range of diameters, including 800 μm , 200 μm , 150 μm , 100 μm , and 75 μm . The pinhole is mounted at the conjugate position of the presumed target spot in the sample. Its size controls the amount of fluorescence light detected from the target spot. A smaller pinhole can reject off-target light more efficiently. However, the detected signal will also be weaker, reducing the signal-to-noise ratio. Therefore, a trade-off needs to be made between the spatial filtering effect and the signal-to-noise ratio.

When investigating the effect of spectral filtering, a filter holder is placed immediately after the 532-nm long-pass filter, as shown in **Fig. 3.1**. The enhancement factor is determined with an empty holder first, which serves as the control experiment. Then the holder is loaded with two bandpass filters, i.e., 618 ± 25 nm and 650 ± 30 nm, sequentially, and the enhancement factor is determined again. Note that the passband of the first filter matches the fluorescence peak of

the purple fluorescent particles, while that of the second filter matches the sideband of the purple fluorescent particles. As the passband redshifts from one filter to the other, the relative fluorescence contribution from the bottom layer increases, thus promoting wavefront shaping performance for depth-sensitive measurements from the bottom layer. However, the overall fluorescence intensity would decrease in this case resulting in a lower signal-to-noise ratio. Therefore, a trade-off must be made between the spectral filtering effect and signal-to-noise ratio, similar to spatial filtering.

3.3. Experimental Results

3.3.1. Wavefront Shaping with Spatial Filtering

A two-layered phantom sample is used in this study to facilitate the observation of fluorescence from each layer, as shown in **Fig. 3.2(A)**. **Fig. 3.2(B)** indicates the individual fluorescence spectra of two fluorophores used, i.e., R6G and purple-colored fluorescent microparticles. Without a pinhole, it can be seen in **Fig. 3.2(C)** that the enhancement factor is relatively low (around 110%) after wavefront optimization. Moreover, the PMT reading appears random in this case, as shown in **Fig. 3.2(D)** during optimization. After a 200- μm pinhole is placed in the position conjugated to the supposed focus in the sample to reject out-of-focus light, a much more significant enhancement factor (around 350%) is observed, as shown in **Fig. 3.2(E)**. Moreover, as shown in **Fig. 3.2(F)**, the PMT Reading steadily increases by 50 iterations. After that, the photobleaching starts to take over, and the PMT reading goes down.

The effect of spatial filtering on the performance of wavefront optimization is evaluated for a series of pinhole diameters, including 800 μm , 200 μm , 150 μm ,

100 μm , and 75 μm , as shown in **Fig. 3.3**. Note that the image of each particle is around 105 μm on the pinhole plane according to that taken by the camera located on the conjugate position. **Fig. 3.3(A)** shows that the contribution from the bottom layer significantly increases when the pinhole diameter decreases from 800 μm to 200 μm or a smaller value, which implies the effectiveness of spatial filtering using a pinhole with a diameter of 200 μm or smaller to reject out-of-focus light for subsequent wavefront optimization. **Fig. 3.3(B)** shows that the enhancement factor increases first as the pinhole size decreases and reaches the maximum at a pinhole diameter of 150 μm . After that, the enhancement factor decreases until there is no enhancement at a pinhole diameter of 75 μm . It is noted that the maximum enhancement is achieved at a pinhole diameter of around 150 μm , which roughly matches the particle image size on the pinhole plane.

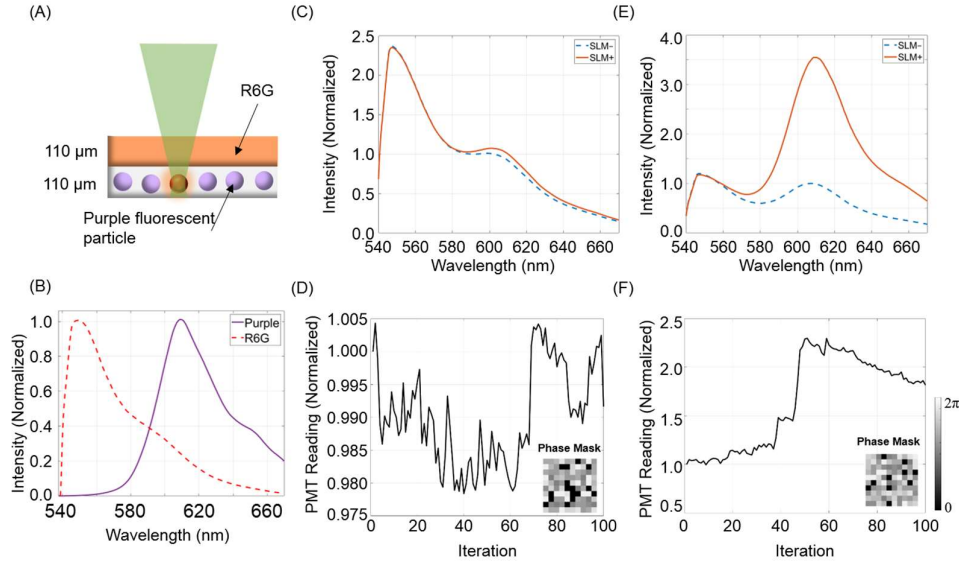


Fig. 3.2. Enhancement of emitted fluorescence with and without a 200-μm pinhole. (A) Schematic of the two-layered sample with R6G in the top layer and purple-color fluorescent particles in the bottom layer. (B) Individual fluorescence spectra of R6G and purple-color fluorescent microparticles. (C) & (E) fluorescence spectra before (blue dash line with the legend “SLM-”) and after (orange-colored solid line with the legend “SLM+”) wavefront optimization detected (C) without and (E) with a 200-μm pinhole, respectively. The spectra have been normalized by dividing each spectrum by the purple-color fluorescent peak before wavefront optimization to facilitate enhancement visualization. (D) & (F) PMT reading during optimization and the resulting phase mask at the end obtained (D) without and (F) with a 200-μm pinhole, respectively. Only a long-pass filter was used to remove excitation light, and no band-pass filter was involved.

3.3.2. Wavefront Shaping with Spectral Filtering

The effect of spectral filtering on the performance of wavefront optimization is evaluated in the cases without a bandpass filter and with a bandpass filter, as shown in Fig. 3.4. Since the fluorescence peaks of the two-layer sample are around 550 nm (R6G in the top layer) and 610 nm (purple-color fluorescent particles in the bottom layer) respectively, spectral filtering with a bandpass filter of 650 ± 30 nm or 618 ± 25 nm increases the fluorescence contribution from the bottom layer as shown in Fig. 3.4(A). Fig. 3.4(C) shows a clear monotonic relation between the

enhancement factor and emission ratio when using the bandpass filter 618 ± 25 nm, demonstrating the positive effect of spectral filtering on the performance of wavefront optimization. In contrast, such a trend is unclear in the cases without any bandpass filter and with the bandpass filter, 650 ± 30 nm, as shown in **Fig. 3.4(B)** and **Fig. 3.4(D)**. It is not surprising to see an unclear increasing trend in enhancement factor in **Fig. 3.4(B)** because fluorescence for guiding wavefront optimization can come from both layers without any bandpass filter. It is also worth noting in **Fig. 4(D)** that the enhancement factor seems more random when using a 650 ± 30 nm bandpass filter than in the other two cases. This can be most likely attributed to the relatively low signal-to-noise ratio in this case because the passband of this filter covers only the tail region of the fluorescence peak in the bottom layer. Consequently, the enhancement factor is influenced, as discussed in the discussion section.

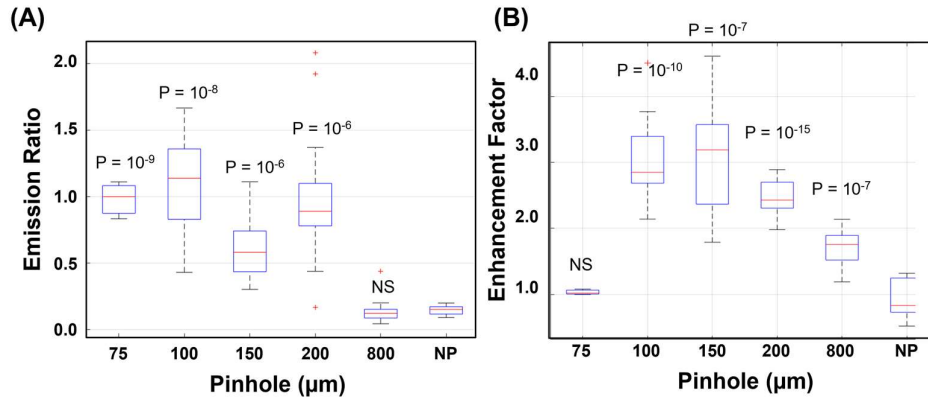


Fig. 3.3. Effect of spatial filtering using pinholes on the performance of wavefront optimization. (A) Emission ratio as a function of pinhole diameter. (B) Enhancement factor as a function of pinhole diameter. Only a long-pass filter is used to obtain the data. The p-value is calculated from the two-sided t-test on the difference between each experimental group with a different pinhole diameter and the control group with no pinhole. NS: Not significant. NP: No pinhole.

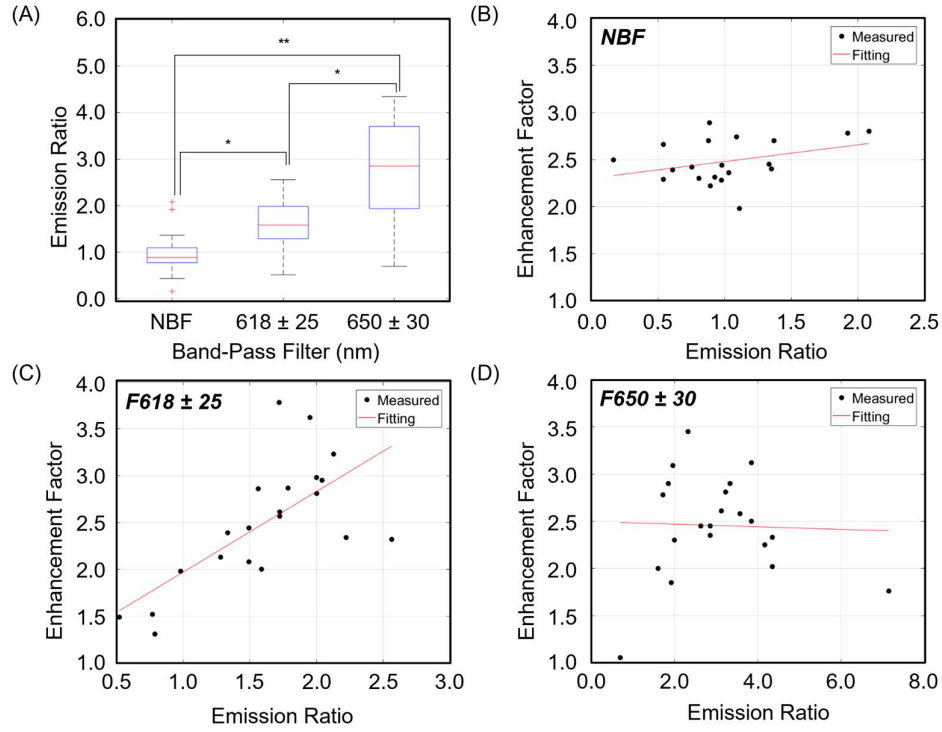


Fig. 3.4. Effect of spectral filtering using band-pass filters on the performance of wavefront optimization. (A) Emission ratio as a function of the central wavelength of the band-pass filter. Enhancement factor as a function of the emission ratio in the cases of (B) no band-pass filter, (C) with a band-pass filter 618 ± 25 nm (D) with a band-pass filter 650 ± 30 nm. A 200-μm pinhole is used to obtain these data. The p-value in (A) is calculated from the two-sided t-test on the difference in emission ratio between each pair of groups. NBF: No Band-pass Filter. **P < 10⁻⁹; *P < 10⁻⁵.

3.4. Discussion

3.4.1. *Effects of Spatial and Spectral Filtering*

Spatial and spectral filtering can effectively improve the enhancement factor, as illustrated in **Fig. 3.2**, **Fig. 3.3**, and **Fig. 3.4**, respectively. The positive role of spatial and spectral filtering in feedback-based wavefront shaping can be attributed to the fact that both filtering techniques increase the relative contribution from the target layer to the feedback signal during wavefront optimization by spatially and spectrally rejecting off-target fluorescence light, which is similar to the roles of the pinhole and bandpass filter in confocal fluorescence microscopy. Spatial filtering with a pinhole works if the target depth is not too large and the guiding light is still detectable, usually within a few scattering mean free path [108]. Spectral filtering can work for depth-sensitive fluorescence spectroscopy in epithelial tissue since it is well-known that endogenous fluorophores in the epithelium and stroma of epithelial tissues are different, with distinctive peaks [109]. Applying these techniques in an actual tissue requires that feedback-based wavefront shaping is faster than speckle correlation in tissues [70, 100], which is currently an active field of research.

3.4.2. *Signal-to-Noise Ratio (SNR)*

Another critical factor in optimization is the feedback signal's signal-to-noise ratio (SNR). Since the optimization relies on whether the feedback signal increases or decreases with the change in wavefronts, it is crucial for the feedback signal to possess a reasonable SNR for stable optimization; if the SNR is too small, the optimization may not be as effective, as demonstrated in **Fig. 3.3** and **Fig. 3.4(D)**. In **Fig. 3.3**, the increasing trend in enhancement factor with a decrease in pinhole size changes when the pinhole is smaller than 150 μm , and there is nearly no

enhancement for a pinhole size of 75 μm , which the poor SNR can explain these cases. Similarly, the increasing trend in enhancement factor with an increase in emission ratio, as shown in **Fig. 3.4(D)**, is supposed to be more significant than that in **Fig. 3.4(C)** if only considering that a greater portion of detected fluorescence light comes from the target layer with the filter $F650 \pm 30 \text{ nm}$ compared with the other filter $F618 \pm 25 \text{ nm}$. However, this trend is not seen, most likely due to the poor SNR of the feedback signal, as the filter $F650 \pm 30 \text{ nm}$ can only catch the weak tail of the fluorescence peak from the target fluorophore. This SNR issue can be much more severe in biological tissues from which the endogenous fluorescence signal is weaker than phantoms samples or exogenous contrast agents. This suggests that using a pulsed laser as the excitation source to achieve a better SNR in the feedback signal may be desirable in future tissue measurements to achieve more significant enhancement and faster optimization.

3.4.3. Photobleaching Artifact

One issue encountered in this study, also common in fluorescence spectroscopy, is the photobleaching issue of fluorophores, which makes it difficult to reach the maximum enhancement [39]. In this study, the photobleaching rate is estimated by monitoring the emitted fluorescence of an individual particle over time with a range of incident powers. A laser power of around 10 to 40 μW yields minimal photobleaching and decent SNR and thus is used during wavefront optimization. With such an incident power, the emitted fluorescence drops only moderately during optimization. The fluorescence intensity is high enough to be measured even by an sCMOS camera for successful optimization. However, it is anticipated that photobleaching may become increasingly severe as the ongoing optimization tends to increase the laser power density on the target particle. One possible

solution for reducing photobleaching is dynamically adjusting the laser power during optimization. Higher laser power can be used in the initial period of wavefront optimization to achieve enhancement before severe photobleaching occurs. Then the laser power can be reduced to slow down photobleaching but is still high enough to yield detectable fluorescence. The fluorescence reading can be numerically scaled up to compensate for its drop due to the laser power reduction. Indeed, using a pulsed laser source could be another solution, as mentioned earlier.

3.4.4. Continuous Sequential Algorithm

Although the continuous sequential algorithm takes longer than several other algorithms, such as the optimal steepest ascent algorithm, it has certain advantages and is suitable for this study. First, the continuous increase in intensity can improve the signal-to-noise ratio for proper signal enhancement with a weak feedback signal. Secondly, compared to the faster optimal steepest ascent algorithm method we evaluated, the continuous sequential algorithm can achieve a higher signal enhancement factor at the end. The continuous sequential algorithm is a proper choice for a static sample such as the one used in the study with a long speckle correlation time [70].

3.4.5. Homogenous Samples and Discrete Targets

One significant difference between phantom samples here (as well as in most other papers about wavefront shaping) and actual tissue samples is the distribution of fluorophores. While the distribution of endogenous fluorophores in tissues such as NADH (reduced nicotinamide adenine dinucleotide) and FAD (flavin adenine dinucleotide) [110] is relatively dense and continuous in cells, the target purple fluorescence particles in this study are discrete and sparse. The discrete and sparse distribution of the target fluorescence particles with adequate distance between any

pair of them artificially reduces the sample volume contributing to the feedback signal and improving the enhancement. This implies that the enhancement can be worse in real tissue samples because the sample volume contributing to the feedback signal can be much more prominent due to the proximity of surrounding fluorophore molecules. We evaluate a phantom sample with dissolved Rhodamine 6G (R6G) as the fluorophore in PDMS and could not achieve any significant enhancement even with pinholes. The distribution of R6G in this sample is continuous since R6G is well dissolved before the PDMS setting. It is anticipated that one should be able to observe fluorescence enhancement when the pinhole size is sufficiently small; however, the challenge lies in providing the feedback fluorescence signal with a sufficient SNR. This could be achieved with a system equipped with a pulsed laser and a sensitive detector.

3.5. Conclusion

The long-term goal of this study is to apply wavefront shaping to tissue imaging. The heterogeneous structure of an actual tissue could be imaged by sequentially scanning the target spot, which is conjugate to the pinhole, throughout the tissue. During scanning, the size of the pinhole can be varied to achieve the trade-off between the spatial resolution and the signal-to-noise ratio. The spectral filter can also be switched to match various target fluorophores. Some weaknesses of the current technique that need to be addressed include the slow speed and low sensitivity. While the former deficiency prevents wavefront shaping from being applied in real tissue with a short speckle correlation time, the latter weakness limits the maximum imaging depth. We are currently working on these two weaknesses by modulating the signal out of the target spot to suppress that from

the surrounding region, which could bring this technique closer to real tissue imaging.

We demonstrate that spatial and spectral filtering can significantly improve the performance of depth-sensitive fluorescence spectroscopy assisted by feedback-based wavefront shaping in tissue-like scattering phantoms. The two filtering techniques work by effectively increasing the relative contribution from the target layer to the feedback signal during wavefront optimization through spatially and spectrally rejecting off-target fluorescence light, which is substantially similar to the role of time or coherence gating. When the filtering techniques are applied, a maximum of three-fold enhancement factor in fluorescence contribution from the target layer is observed, which is in contrast to nearly no enhancement in the case of no filtering. These two techniques, integrated with feedback-based wavefront shaping, can improve depth-sensitive fluorescence spectroscopy for the characterization of layered structures such as epithelial tissues or tablets, in which the creation of an external guide star is challenging or not allowed.

Chapter 4

Wavefront Shaping with AOD Modulation

4.1 Introduction

Feedback signal intensity and time spent on each iteration are two crucial factors for feedback-based wavefront shaping to work well. Compared to a spatial light modulator (SLM, 60 Hz), digital micromirror device (DMD, 22 kHz) [65], and deformable mirror (DM, 1 kHz) [111], an acousto-optical deflector (AOD) [112] has a higher refreshing rate (> 500 MHz) that can benefit those applications with dynamic changes. For instance, AOD has been successfully used in high-speed scanning [112-114], which is faster than conventional methods due to its high deflection speed with no moving components involved (note that mechanical motion would require time to be stabilized). Laser beam scanning with high temporal resolution and random access is very suitable for biomedical applications such as multiphoton confocal microscopy [115, 116], optical tweezer [116-121], and axial scanning [122]. However, compared to a galvanometer mirror, the deflection angle of an AOD is relatively small ($\sim 2.5^\circ$) for a model with a bandwidth of 50 MHz. It will limit the field of view (FOV) when a large scanning area is required with a fine resolution.

The acousto-optic effect of the AOD enables high-speed spatiotemporal and spectral modulation of the input light. Both precise amplitude and phase modulation of coherent laser light are feasible and valuable for high-speed wavefront shaping [122, 123]. Unlike direct laser light manipulation, the AOD generates frequency shifts and phase changes in diffracted light with a driving radio frequency (RF) signal, which can be exploited to detect the feedback signal more effectively for wavefront shaping. This chapter presents feedback-based wavefront shaping with AOD phase modulation and discusses its performance in different conditions.

4.2 Principle and Methodology

4.2.1 *Phase/amplitude Modulation of Light with Acoustic Wave*

Ultrasonic wave propagation changes the crystal's refractive index inside an AOD, which induces the Doppler effect on a laser beam traveling across and generates frequency shifts. The crystal with varying refractive index induced by the ultrasonic waves behaves like a dynamic grating [124] that deflects the laser to an angle ($\Delta\theta$) relative to the incident light. The angle depends on the driving frequency (Δf), the wavelength (λ) of the input laser, and the acoustic speed (v_s) in the crystal in the Bragg regime that yields high efficiency in light diffraction [19].

$$\Delta\theta = \frac{\Delta f \cdot \lambda}{v_s} \quad \text{Eq. (4.1)}$$

The ultrasound wave modulates the light diffracted by the AOD in terms of several wave properties, including frequency, amplitude, and phase. Therefore, the phase and amplitude of the output laser beam will carry information from the ultrasound $A_{AOD}e^{i(\omega_{AOD}t+\varphi_{AOD})}$ as shown in **Equation (4.2)** [123]

$$E_{out} = A_{Laser}A_{AOD}e^{i[(\omega_{Laser}+\omega_{AOD})t+(\varphi_{Laser}+\varphi_{AOD})]} \quad Eq. (4.2)$$

where A is the amplitude, ω is the angular frequency, φ is the phase, and the subscript indicates the source of each term. After AOD modulation, the deflected laser light (E_{out}) frequency is shifted by ω_{AOD} , and the phase is increased by φ_{AOD} . Because the deflection efficiency is affected by the power of acoustic waves, the amplitude of deflected light can also be modulated by A_{AOD} . If there is a multiplexed signal with different frequencies, multiple diffracted laser beams ($E_{out,1}, E_{out,1} \dots E_{out,N}$) will be generated. For instance, when the laser beam is diffracted by two ultrasonic waves with different angular frequencies (ω_{AOD1} and ω_{AOD2}) and phases (φ_{AOD1} and φ_{AOD2}), two laser outputs will be generated, each carrying the respective frequency and phase.

$$E_{out,1} = AA_{AOD} \sin[(\omega_{Laser} + \omega_{AOD1})t + (\varphi_{Laser} + \varphi_{AOD1}) - \mathbf{k} \cdot \mathbf{r}_1] \quad Eq. (4.3)$$

$$E_{out,2} = AA_{AOD} \sin[(\omega_{Laser} + \omega_{AOD2})t + (\varphi_{Laser} + \varphi_{AOD2}) - \mathbf{k} \cdot \mathbf{r}_2] \quad Eq. (4.4)$$

where the last terms in the exponents represent the phase contribution from spatial displacement. Hence, when the two diffracted light beams generate spatiotemporal interference, the interference signal intensity (I) can be written as **Equation (4.5)**.

$$\begin{aligned} I(k, t) = & |E_1(k, t)|^2 + |E_2(k, t)|^2 \\ & + 2|E_1(k, t)||E_2(k, t)|\cos[(\omega_{AOD1} - \omega_{AOD2})t + \varphi_{AOD1} - \varphi_{AOD2} \\ & + \mathbf{k} \cdot (\mathbf{r}_2 - \mathbf{r}_1)] \quad Eq. (4.5) \end{aligned}$$

The terms involving laser frequency and the initial phase are canceled out. The difference between the AOD frequencies turns into a “beat frequency” ($\Delta\omega = \omega_{AOD1} - \omega_{AO}$). Since the interference signal intensity ($|E_1(k, t)||E_2(k, t)|e^{i((\Delta\omega t + \Delta\phi))}$) is time dependent on the order of megahertz, a single-pixel detector with high bandwidth can detect it. Due to the propagating nature of the acoustic wave in an AOD, the phase carried from the acoustic wave to the output light in wavefront shaping will change periodically. Consequently, the output light has a frozen phase only within a short moment, and the moment will repeat in every period of the AOD driving signal. Therefore, different from the time-invariant phase modulation using an SLM, the dynamic wavefront shaping of AOD only provides an optimized phase within a short time window of nanoseconds [75]. Like the effect of low-coherence interferometry such as OCT, when the beat signals of different frequencies are all in phase, a peak will be generated in the time domain as temporal focusing. Therefore, a time-gated signal or timing-controlled light source is required to get a diffracted laser light with stable phase modulation or a frozen phase. For this purpose, a pulse laser [123] or AOM-modulated light [75] is commonly used to synchronize the timing of the optimized phase and isolate the desired signals. In contrast, a continuous-wave laser is used in our study by utilizing beat signals created by AOD modulation of the laser light. Instead of taking the time-domain signal as feedback, we use the amplitude of the modulation frequency in the frequency domain as feedback for optimization, which works just as effectively without requiring a time-controlled light source.

4.2.2 Generation of Multiple Diffracted Laser Beams

We use a function generator to produce RF signals to drive AOD (AODF 4090-7, G&H, UK) after an RF amplifier. The propagating RF wave generates an acoustic wave in the AOD crystal, forming a dynamic grating structure [124]. The laser beam passing through the crystal will be diffracted at a certain angle following Bragg's law, which states that the diffracted angle is determined by the frequency and speed of the acoustic wave propagating inside the crystal. Since the crystal's speed and other parameters are considered constant during the experiment, we can generate a laser beam diffracted at a certain angle with the desired frequency shift by applying an RF signal to the AOD. Furthermore, by taking advantage of the feature, we can generate a series of angles using multiple RF signals. **Fig. 4.1** shows one example of the diffracted light pattern after the AOD when using 41 frequencies from 60 to 100 MHz, each carrying a different frequency shift. The total efficiency of the AOD is over 70 % and can go up to 85 % for the number of frequencies varying from 1 to 200 (**Fig. 4.2**). The relative phase and amplitude of each RF frequency component can be controlled to modulate coherent laser light and alter the beat signal (interference) in the RF region.

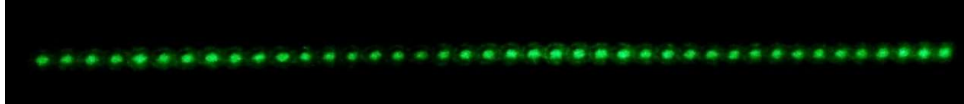


Fig. 4.1. The 1D diffracted light pattern is generated by a single AOD driven by the superposition of 41 RF components (60 – 100 MHz).

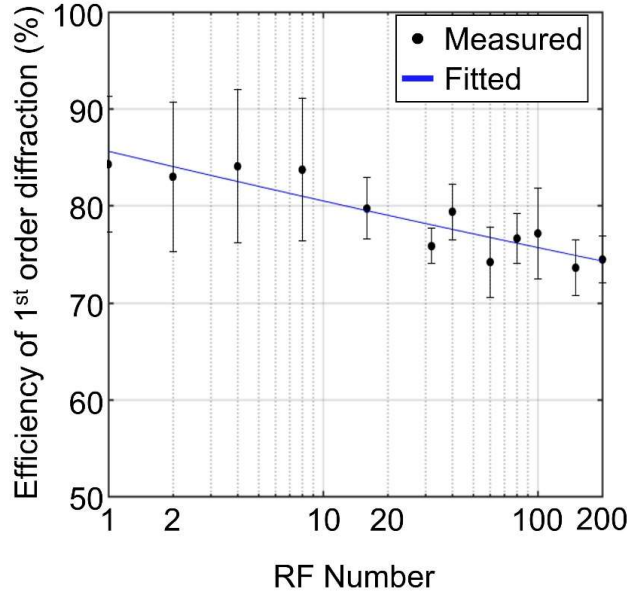


Fig. 4.2. The first-order diffraction efficiency with different numbers of driving frequencies in an AOD (AODF 4090-7; G&H).

4.2.3 Feedback Signal Selection

To apply the optimal steepest ascent (OSA) algorithm (refer to **Chapter 2, section 2.4.2**), there are two approaches to selecting the feedback signal to determine the optimal phase for each RF component. One approach uses all RF components as the feedback signal, while the other approaches select one fixed RF component as the feedback signal. For the first approach, the phase for each RF component needs to be measured and calculated separately. In this study, the beat frequencies are generated by the interference between the part of the incoming light and the first-order diffracted light when using RFs of 60, 61, ..., and 100 MHz for the AOD. Therefore, the first approach requires the estimation of optimal phases to be performed 40 times for RFs of 60, 61, ..., and 100 MHz. In the second approach, all RF components can generate a common beat frequency component, thus carrying information about all components that need

to be used. In this study, the beat frequencies generated by the interference among the 1st-order diffracted light beams include 1, 2, 3, ..., 40 MHz when using RFs of 60, 61, ..., and 100 MHz for the AOD. The amplitude of the 1 MHz beating frequency is the strongest in this case since the difference between 39 pairs of RFs could generate this frequency (60 and 61, 61 and 62, ..., 99 and 100 MHz). The strongest signal in this frequency can be advantageous for a highly scattering sample since the emission signal from the sample can be very weak.

In the second approach, in addition to the first-order diffracted light, we also use part of the incoming light split by the beam splitter as the reference beam. The beat frequency components in this setup will come from the interference among the first-order beams and the interference between the 0th-order and 1st-order beams, which cover the ranges of 1, 2, 3, ..., 40 MHz and 60, 61, 62, ..., 100 MHz, respectively. Unlike the former beat frequencies in which multiple pairs of RFs can generate each beat frequency, each of the latter frequencies is unique. Therefore, every beat frequency is independent and can serve as an individual feedback signal. With multiple individual feedback signals, we only need to measure the light signal once to calculate the optimized phase.

4.2.4 Sample Preparation

- ***TiO₂ Phantoms***

TiO₂ and ZnO are commonly used for scattering medium since they have a very high refractive index. We mixed the TiO₂ (1.5 μm particle size) with PDMS to fabricate solid homogeneous phantoms. The concentration of TiO₂ is varied to control the reduced scattering coefficient, and transport mean free path. The

transport mean free path is estimated by use of its relationship with the inverse transmission according to **equation (4.6)** [57, 125]

$$\frac{1}{T} = \frac{L + Z_{e1} + Z_{e2}}{\ell + Z_{e1}} \quad Eq. (4.6)$$

where $\frac{1}{T}$ is the inverse of total transmission. L is the sample thickness, and ℓ is the transport mean free path. Z_{e1} and Z_{e2} are the interior and exterior extrapolation lengths calculated from the effective refractive indices, while R is the reflectivity [17].

$$Z_e = \frac{2}{3} \left(\frac{1 + R_2}{1 + R_1} \right) \quad Eq. (4.7)$$

We measured the inverse transmission values of samples with different thicknesses. The transport mean free path was calculated from the linear fitting of the resulting plot (inverse transmission vs. sample thickness) with the equation $y = 0.0174x + 1.6297$. The estimated slope can be used to derive the transport mean free path, which was found to be $\ell = 27 \mu\text{m}$ with an effective refractive index of 1.4 for the data in **Fig. 4.3**.

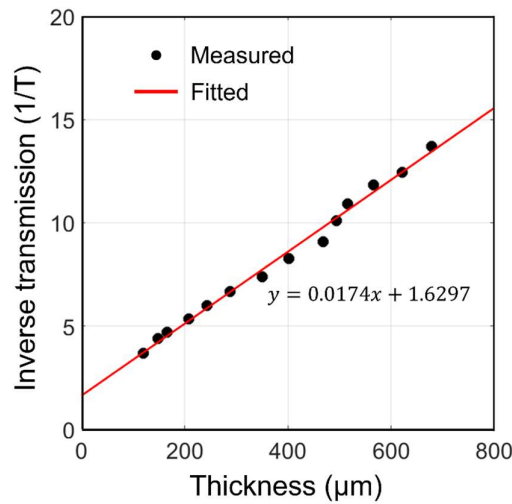


Fig. 4.3. Relation between the inverse transmission and the sample thickness of a TiO₂ phantom.

4.3 Experimental Results

4.3.1 Focusing Laser Light through TiO_2 Phantoms

The scattering phantom used here is made of a 400- μm -thick layer of TiO_2 and PDMS with a transport mean free path of 424 μm at 532 nm. We use the 1-MHz AC signal as feedback for the OSA. With feedback-based wavefront shaping, the magnitude of the signal component at 1 MHz can be enhanced, as shown in **Fig. 4.4 (C-D)**. To focus light onto the region matching the size of one laser speckle to maximize the performance of wavefront shaping, we use an avalanche photodetector (APD) with a bandwidth of 400 MHz and a sensor with a diameter of 200 μm diameter located 14 cm away from the sample (**Fig. 4.4(A)**). The sensor of the APD is used as a spatial filter to isolate the signals from the region close to speckle size. In this experiment, we use the AOD to optimize the light on the target at first and then move the photodetector along the x-axis to measure signal changes. This can help us analyze the resolution on the imaging plane and the focusing capability on the target point. **Fig. 4.4(C)** shows that only the focal spot has the highest enhancement factor, and other locations do not show significant changes. The full width at half maximum (FWHM) of the peak ($\sim 110 \mu\text{m}$) is similar to the size of the APD sensor (200 μm), as shown in **Fig. 4.4(B)**. In this case, a single-pixel detector with a smaller sensor can potentially achieve a smaller focal spot size but could suffer from a lower signal-to-noise ratio, which would lead to poorer signal enhancement. Therefore, the trade-off between the focal spot size and signal-to-noise ratio is essential. In summary, the result indicates that the phase modulation of RF signals driving the AOD can indirectly change the wavefront of laser light and enhance constructive interference in a

small target region. The increasing constructive interference can be observed as the beat signal detected by a photodetector.

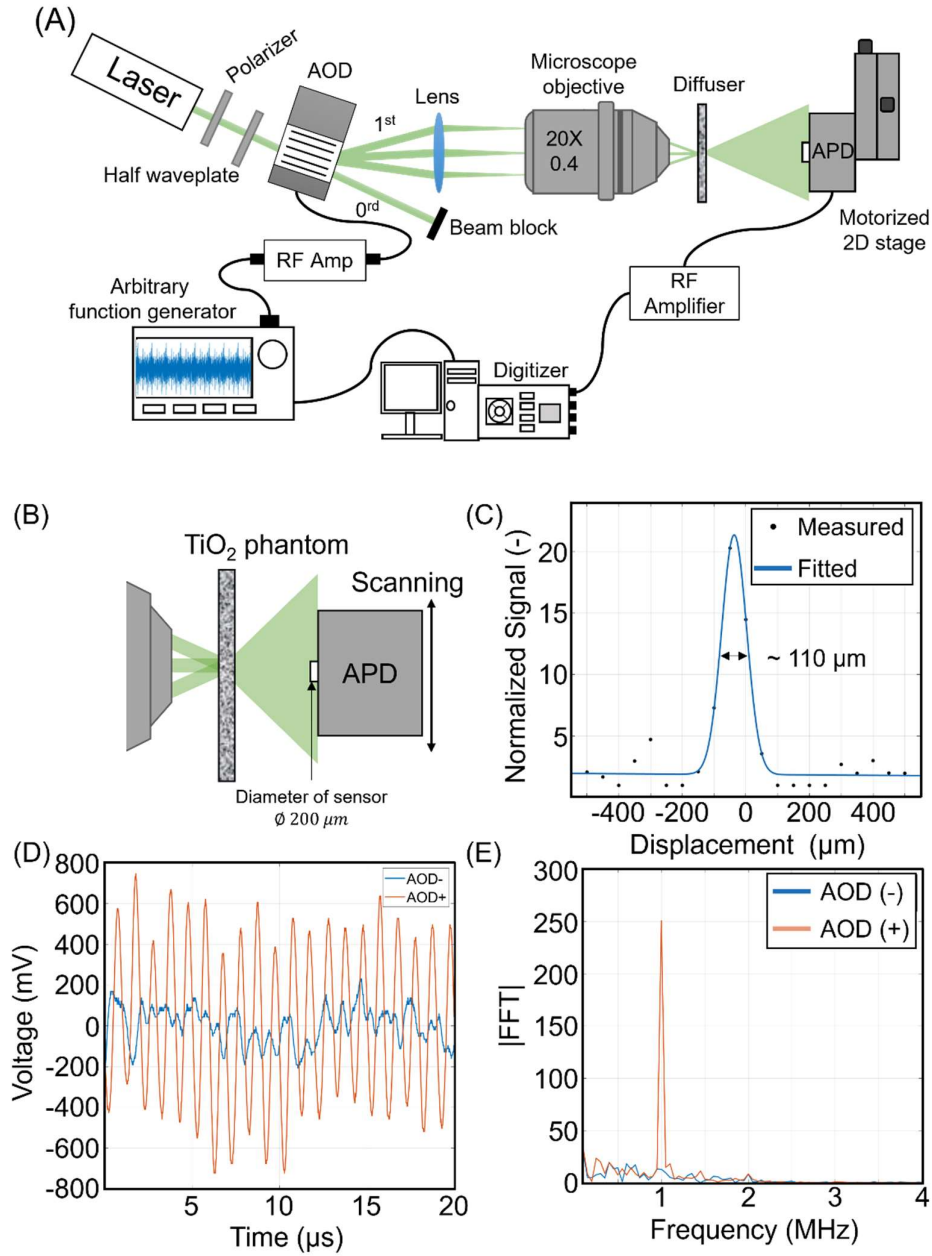


Fig. 4.4. Focusing light through a scattering TiO_2 phantom. (A) Schematic of the optical setup. (B) APD detector is located 14 cm behind the TiO_2 phantom for feedback signal measurements. (C) Enhancement factor along the x-axis after AOD modulation. The enhanced signal after wavefront shaping in (D) the time domain and (E) the frequency domain when using the magnitude of the 1-MHz component as the feedback.

4.3.2 Enhancement Factor and Laser Power

The AOD's diffraction efficiency is affected by the ultrasound wave energy generated by RF driving signals, which may influence enhancement performance. Therefore, to understand this factor better, we compare the enhancement performance by monitoring the level of laser power change and the enhancement factor. In this experiment, the enhancement factor of the AOD modulation can be calculated from the 2D imaging by using the ratio of peak intensity (I^*) to average background intensity (I_0), as shown in **Fig. 4.5(A)**. We observed that the total laser power of the first-order diffraction after AOD is also increased by around 30% after wavefront shaping, as shown in **Fig. 4.5(C)**. Compared to the drastic signal enhancement ($> 700\%$) using 64 degrees of freedom (**Fig. 4.5(B)**), the influence of power increase is small and can be ignored.

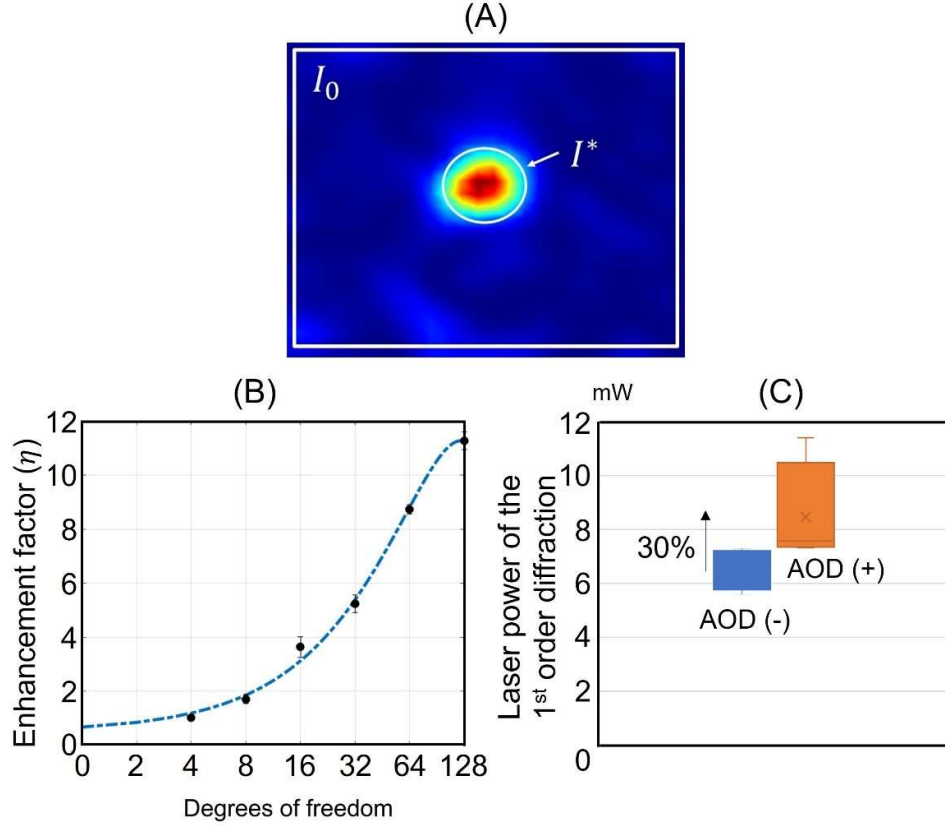


Fig. 4.5. Effects of degrees of freedom and laser power on enhancement factor. (A) 2D imaging of focus spot after wavefront shaping. (B) The enhancement factor is positively correlated to the degrees of freedom. (C) AOD modulation increases the power of the output laser beam after AOD by around 30%.

4.3.3 Phase and Amplitude Modulation

Compared to phase-only LCOS-SLMs or amplitude LCOS-SLMs, AOD can simultaneously modulate the laser light in both amplitude and phase. Referring to **Equation (2.3)** in **Section 2.1**, the theoretical enhancement factor $\eta = \alpha(N - 1) + 1$ is determined by the coefficient factor α (phase-amplitude modulation: $\alpha = 1$; phase-only modulation: $\alpha = \pi/4$). In this experiment, we optimize the phase first and then the amplitude at each frequency sequentially to find out if optimizing an extra parameter, i.e., the amplitude, would considerably improve enhancement. The scattering phantom used here was a 300- μm -thick

layer of TiO₂ phantom in PDMS with a transport mean free path of 500 μm at 532 nm. Thirty-two radio frequencies with the smallest beat frequency of 0.5 MHz are used to drive the AOD. **Fig. 4.6** indicates that in most situations, optimizing amplitude as an extra parameter can slightly ($\sim 20\%$) increase the enhancement factor, which is similar to the theoretical difference ($\sim 26\%$). However, optimizing the extra parameter doubles the time required to complete wavefront optimization. Therefore, there is a trade-off between more significant enhancement and faster optimization.

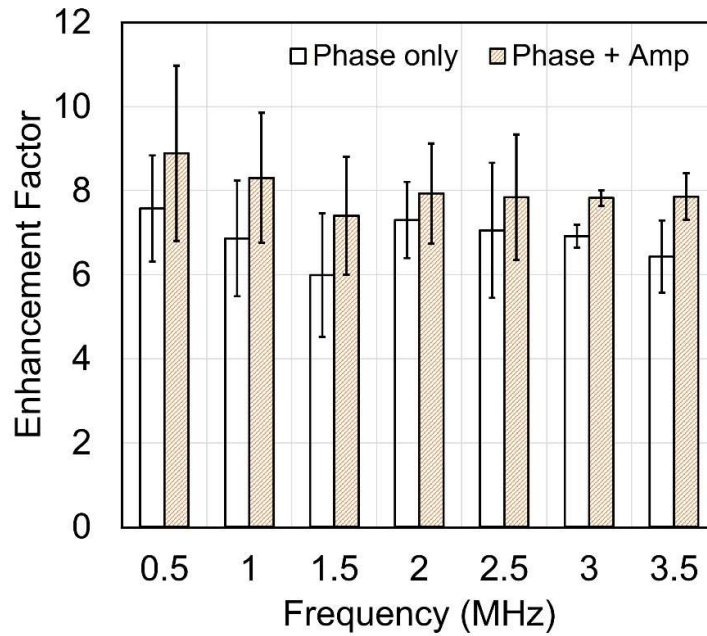


Fig. 4.6. Comparison of phase and phase-amplitude modulation. Optimizing amplitude as an extra parameter can improve enhancement.

4.3.4 Comparison with SLM Modulation

Many factors, such as dark currents, environment noise, and stray light, can influence the quality of the commonly used DC feedback signals. For the AOD modulation, the interference of multiple light beams on the target position generates a beat signal with a specific radio frequency. The signal encoded with a particular frequency differs from the background that a detector with a high-bandwidth photodetector can measure. Therefore, compared with DC signal measurements, AC signal measurements can isolate the target signal within a specific frequency range from a noisy background. Isolating the target signal can lower the power threshold required for wavefront shaping to work by one order of magnitude or more (**Fig. 4.7**). The results show that the AC-based modulation has the advantage of resisting noise during feedback-based wavefront shaping, which makes it more suitable for low light enhancement.

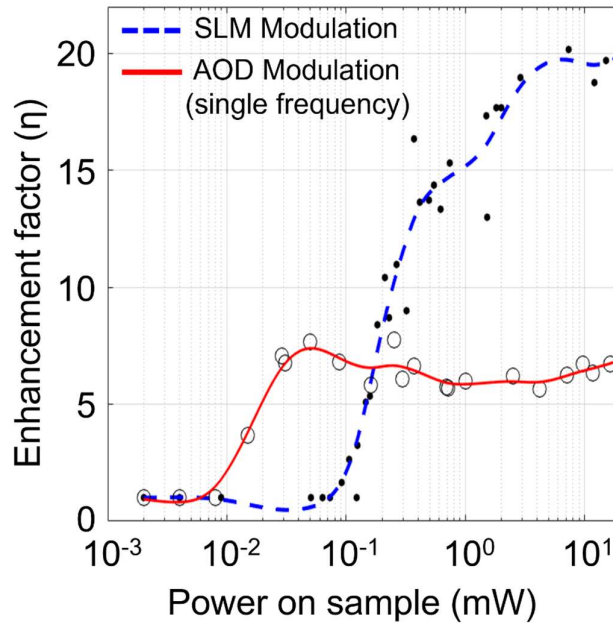


Fig. 4.7. Comparison of the SLM and AOD modulation with different incident powers.

4.3.5 *Enhancement of Fluorescence Spectra*

Fluorescence, similar to the SLM experiment in **Chapter 3**, can be used as the feedback signal for wavefront shaping. Since the 1-MHz signal is used as feedback and targeted for enhancement, the optimized signal has a frequency of 1-MHz. Therefore, we need a single detector with high bandwidth to measure the signal. However, most commercial spectrometers use 1D or 2D cameras as detectors that are not fast enough to capture these high-frequency signals. We built a custom scanning spectrometer to measure the spectrum by integrating a diffraction grating, a galvo mirror, and a PMT. The wavelength was shifted by scanning the mirror at different angles. The voltage used to drive the scanning mirror can be converted into wavelength with proper calibration, which involves using a common light source and a commercial spectrometer. We tested various fluorophores in TiO₂ phantoms with different emission spectra. As shown in **Fig. 4.8**, the results show that feedback-based wavefront shaping can enhance the entire spectra accordingly.

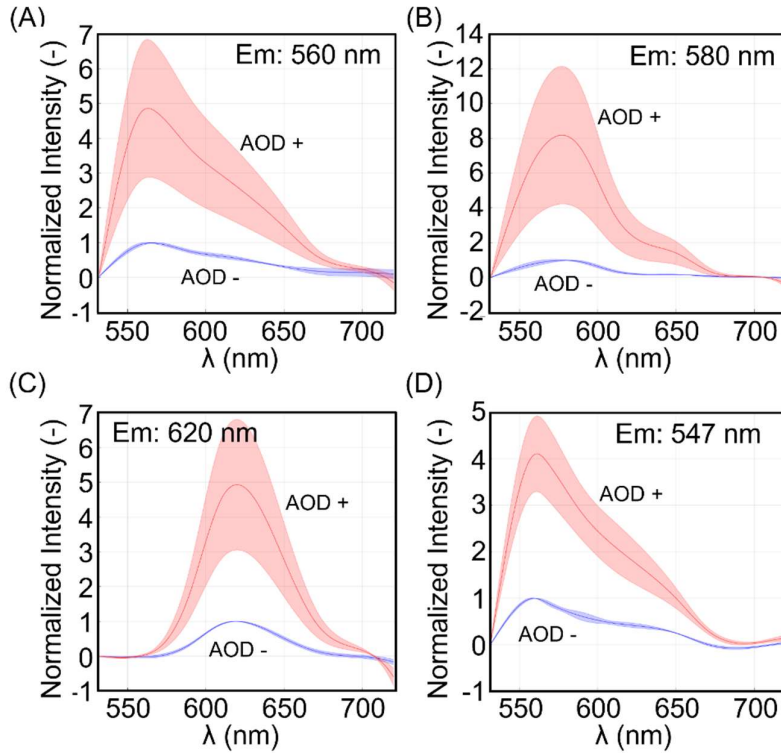


Fig. 4.8. Emission spectra of (A) Nile Red fluorescent particles (B) Pink-colored fluorescent particles (C) Purple-colored fluorescent particles (D) Quantum dots phantoms before (labeled as “AOD –”) and after (labeled as “AOD +”) wavefront shaping.

4.3.6 Fluorescence Enhancement: Laser-Guided vs. Fluorescence-Guided Wavefront Shaping

Fluorescence signals from fluorophores in scattering media can be enhanced, as shown in **section 4.3.5**. Both excitation light and emission light can be used as feedback for iterative optimization. Compared to fluorescence emission, scattered laser light has much higher power and a higher signal-to-noise ratio. However, laser as feedback is a type of indirect optimization since the goal is to enhance the fluorescence from a target, which may be limited in specific scenarios, e.g., where the target fluorescence offers better localization. Here, we

investigate the advantages of directly using fluorescence as feedback for its enhancement. In this experiment, we use a fluorescent strip as the target by drying up a drop of quantum dots suspension on a semi-transparent weighing paper (**Fig. 4.9(B)**). The fluorescent strip is between two TiO_2 phantoms (**Fig. 4.9(A)**). We then separately perform the wavefront shaping using 532-nm excitation light and 547-nm emitted light as feedback. After wavefront shaping targeted at one location, we make a lateral scan of the sample with a motorized stage for a 1D profile measurement. **Fig. 4.9(C)** shows that wavefront shaping can improve lateral resolution by approximately 73% when using fluorescence as feedback. Moreover, **Fig. 4.9(D)** shows that the improvement in lateral resolution in terms of full width at half maximum (FWHM) when using excitation light as feedback is considerably smaller than when using fluorescence directly as feedback. In short, the result indicates that even though laser light has higher SNR when served as feedback, wavefront shaping using fluorescence directly as feedback is advantageous in resolution when targeting fluorescence.

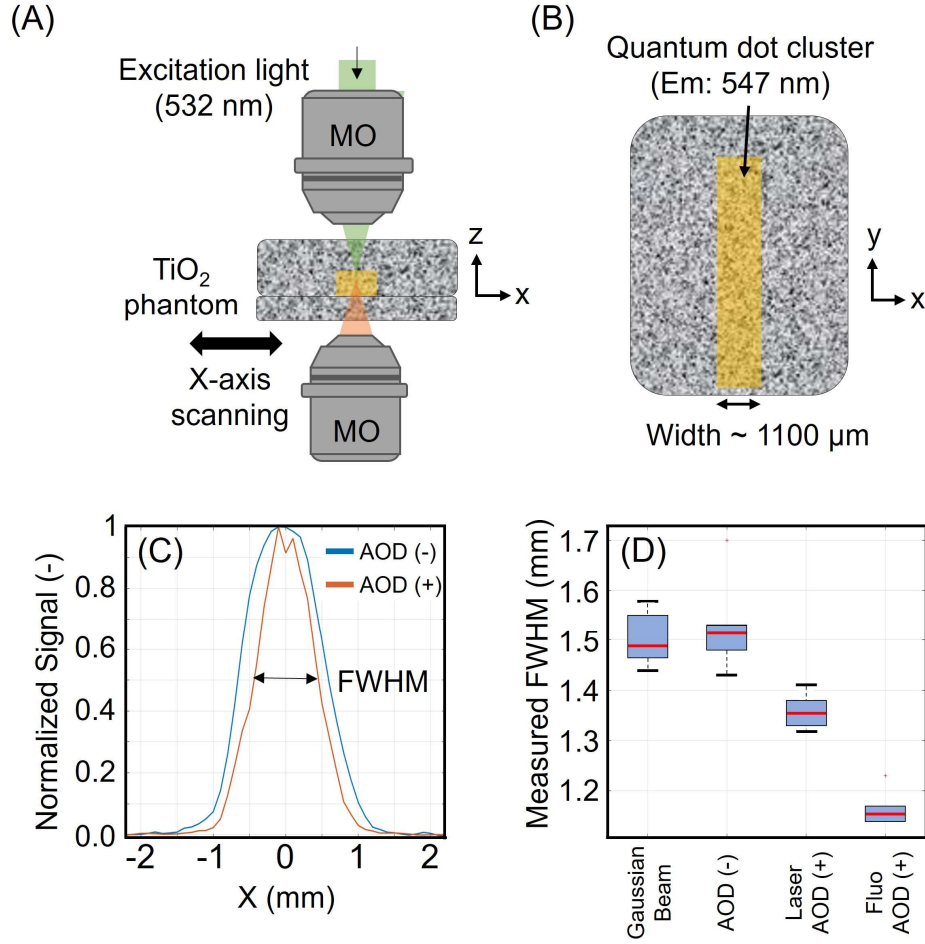


Fig. 4.9. Laser-guided vs. fluorescence-guided wavefront shaping for fluorescence enhancement. (A) TiO_2 phantom is used as the scattering medium; (B) the fluorescent target made by a stripped shape of quantum dot clusters with a thickness of 1.1 mm is put in between for measurements. (C) The 1D fluorescent profile was obtained by scanning the sample along the x-axis. (D) From the 1D profile, the FWHM can be measured for comparison. Gaussian beam: Excitation light without passing through AOD crystal; AOD (-): Excitation light after passing through AOD with random phase; Laser AOD (+): Excitation light with optimized phase modulation by using laser speckle as the feedback for optimization; Fluo AOD (+): Excitation light with optimized phase modulation by using fluorescence as the feedback for optimization

4.4 Discussion

- *AC vs. DC Signal with Single/Multiple Frequency Optimization*

In SLM modulation, the feedback signal is usually detected as a DC signal since a common photodetector is not fast enough to capture the high frequency of light. Unlike the SLM, a critical feature of using AOD for phase modulation is that the modulated signal is time-dependent due to light interference, which presents as an AC signal with radio frequencies. Therefore, when AOD is used to refocus light on the target by wavefront shaping, we will not see considerable DC signal enhancement as captured by an sCMOS camera with a 100-fps rate (using the setup shown in **Fig. 4.4(A)**) as shown in **Fig. 4.10** since it is not fast enough for measuring RF signals. Instead, a fast point photodetector, such as a photodiode with a large bandwidth, will be used to measure reflectance and fluorescence signals.

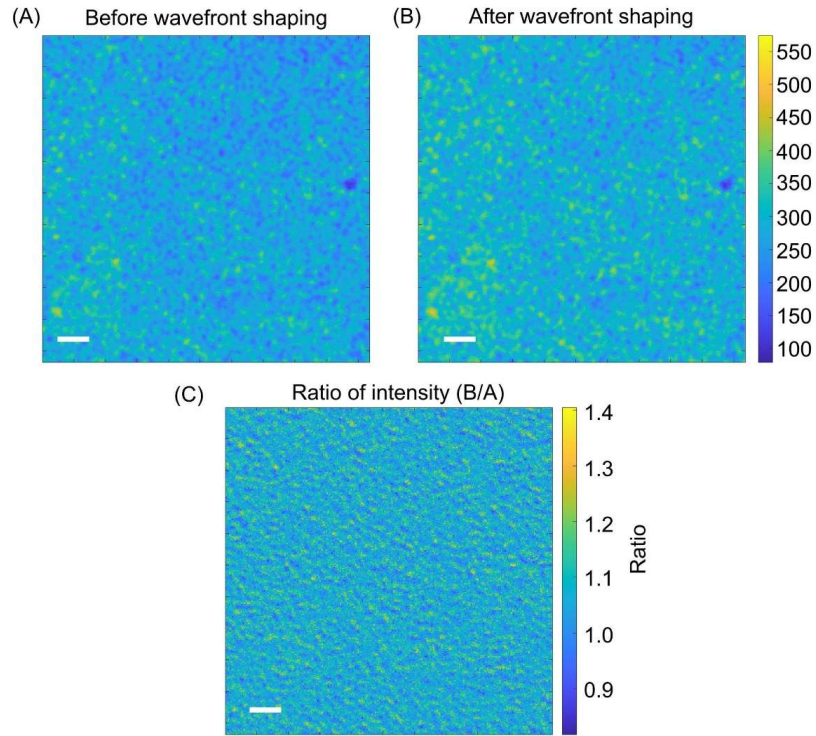


Fig. 4.10. 2D camera image of the target region (A) before and (B) after AOD modulation. (C) The enhancement factor calculated from the two images shows no significant enhancement after wavefront optimization because of slow detection—scale bar: 100 μm .

4.5 Conclusion

In this chapter, we demonstrate that AOD can be used to perform phase and amplitude modulation simultaneously on laser light for feedback-based wavefront shaping. AOD-based wavefront shaping, similar to SLM-based wavefront shaping, can be used for incoherent light enhancement. Compared to DC feedback signals used for SLM modulation, AC feedback signals in AOD modulation have better SNR and can lower the threshold of required light power for effective optimization. Moreover, the high refreshing rate of modulation and AC signal generation of AOD offer potential advantages over SLD in dealing with weak signals such as fluorescence. In **Chapter 5** and **Chapter 6**, we will demonstrate related experimental results and details about the increasing SNR for weak signals and high-speed wavefront shaping for dynamic samples, respectively.

Chapter 5

Feedback-based Wavefront Shaping for Weak Light with Lock-in Beat Frequency Detection

5.1. Introduction

Iterative wavefront shaping [38] has the advantage of improving the signal ratio to the background to overcome the multiple scattering issue for thick samples [102]. In addition, it works for both coherent and incoherent light in nature, which is thus more versatile than those techniques requiring coherent light. Fluorescence, which is much weaker than elastically scattered excitation light, is very challenging to measure in a thick sample with high scattering. Feedback-based wavefront shaping has been developed to enhance fluorescence [39, 42, 45, 78] and Raman signals [79]. However, the SNR of feedback signals affects the signal enhancement of feedback-based wavefront shaping strongly [126]. The method can fail when the feedback's SNR is too low to ensure consistent improvement in each iteration during optimization.

So far, most current feedback-based wavefront shaping techniques are based on direct current (DC) feedback signals and are thus prone to the influence of noise and signal drift, which can render wavefront shaping ineffective. For example, Vellekoop et al. and Li et al. used DC fluorescence as feedback, thus requiring strong fluorescence [10, 11]. Thompson et al. used an SLM in which a

DC Raman signal served as feedback and suffered from low enhancement [12]. Previously we used an SLM with spatial and spectral filtering with a DC signal as feedback [13]. On the other hand, Lock-in detection effectively detects weak signals at a selected frequency from strong background noise. It has been proposed to improve the SNR for fluorescence [127] and photoacoustic signals [128]. For visible light, downscaling the optical frequency to a detectable range is a powerful way to analyze light frequency information. For instance, frequency comb spectroscopy [129] and radiofrequency-tagged emission (FIRE) microscopy [130] measure the light spectrally and spatially by analyzing light signals in the radio frequency region.

Acousto-optical deflector (AOD) has recently been demonstrated as a useful device to modulate light with an acoustic wave for wavefront shaping [75, 76] using digital phase conjugation. The device can be used to modulate the amplitude and phase of light with a refreshing rate [123] higher than most SLM and digital micromirror devices (DMD) [65] at the cost of fewer individual elements. Ultrasound in the crystal of an AOD causes the frequency shift of the incident laser beam due to the Doppler effect. After passing through AOD, a monochromatic light will be diffracted into various angles, and each is encoded with a different radiofrequency. The frequency differences yield beat frequency components when the beams merge and interfere with each other. The beat frequencies are typically in the megahertz range and can be detected using a fast detector, making it possible to apply lock-in amplification in wavefront shaping using AOD.

In contrast to the earlier papers [10-13] using DC feedback-based wavefront shaping, we propose to illuminate a thick sample with multiple light beams, each

shifted by a different radio frequency, generated by an AOD, and measure the resulting AC signal of selected beat frequencies through the sample with a lock-in amplifier as the feedback to significantly improve the feedback's SNR thus extending feedback-based wavefront shaping to weak light. The phase distribution of the driving radio frequency signal for the AOD is optimized for wavefront shaping. It is demonstrated that feedback-based wavefront shaping with lock-in amplification for a single beat frequency component works effectively for weak light like fluorescence and Raman signals in a reflection setup. The technique could be helpful for *in vivo* measurements in which a reflection setup is typically required, and signals are weak.

5.2. Principle and Methodology

5.2.1 Principle

An arbitrary function generator was used to generate signals with multiple frequencies $Q(t) = \sum_{j=1}^N a_j \cos(\omega_j t - \varphi_j)$ to drive the AOD, where a , ω , and φ are amplitude, angular frequency, and phase, respectively. The phases were randomized to prevent the generation of sharp instant power peaks, which could otherwise cause permanent damage to the AOD crystal. After interacting with the dynamic grating generated by the driving signal in the AOD, an array of laser beams with different diffraction angles will be generated, each shifted by a different frequency due to the Doppler effect. The light beams illuminate the sample from a range of angles at the conjugate plane of the AOD. The superposition of multiple laser beams inside the sample forms a speckle pattern on the other side of the sample. Because of the different frequency shifts, the interference among these beams generates signals with various beat frequencies. The resulting alternative current (AC) signal $S(t)$ received by the detector after excluding the direct current component (DC) can be described by

$$\begin{aligned}
 S(t) \propto & \sum_{j=1}^{N-1} |a_{j+1}| |a_j| \cos[(\omega_{j+1} - \omega_j)t - (\varphi_{j+1} - \varphi_j)] + \\
 & \sum_{j=1}^{N-2} |a_{j+2}| |a_j| \cos[(\omega_{j+2} - \omega_j)t - (\varphi_{j+2} - \varphi_j)] + \dots \\
 & \sum_{j=1}^1 |a_{j+N-1}| |a_j| \cos[(\omega_{j+N-1} - \omega_j)t - (\varphi_{j+N-1} - \varphi_j)] \quad \text{Eq. (5.1)}
 \end{aligned}$$

where N represents the total number of driving RF fed to AOD, ω represents the angular frequency, φ represents the phase of each RF component, and the

subscript refers to the index of the RF signal. Totally, there will be $N-1$ different beat frequencies if all RFs are equally spaced. The final AC signal $S(t)$ is the summation of all beat signals. Hence, the signal can be detected by a commercial single-pixel detector with large bandwidth.

It is well known that the summation of multiple sinusoidal functions can yield a sharp peak when they are all in phase. In wavefront shaping as modeled by **Eq. (5.1)**, adjusting the phases of all beat signals makes it possible to match the phases of all beat signals and yield a sharp peak in the time-domain signal (**Fig. 5.3**), which results in the spatiotemporal focusing effect. In contrast, by adjusting the phases of a portion of beat signals with a single frequency in wavefront shaping, only the amplitude of the selected beat frequency component will be mainly enhanced, while the peak is not as prominent in the time-domain signal. The spatiotemporal focusing (optimization involving all beat signals) and proposed lock-in method (optimization involving a portion of beat signals at a selected frequency) are both used in this article.

Fourier transform can calculate the magnitude of each beat frequency component from the detected signal. In this experiment, the driving signal covers a frequency range of 60 - 100 MHz. Totally, we use 64 frequencies (60, 61, ..., 100 MHz and 68.5, 69.5, ..., 90.5 MHz). The beat frequency originating from the interference between beams ranges from 0.5 to 40 MHz. The signal-to-noise ratio decreases as the beat frequency increase from 0.5 to 40 MHz because the number of beams involved in related interference events decreases with the increasing beat frequency. We select a beat frequency of 0.5 MHz or 1 MHz as the feedback because the two components are more substantial than the others. We use a lock-in amplifier to detect the weak signal with a specific frequency.

Furthermore, the magnitude of the 0.5 or 1 MHz beat frequency component serves as feedback. We use the optimal steepest ascent (OSA) algorithm [103, 104] (refer to **Chapter 2, section 2.4.2**) to optimize the wavefront according to the following formula to maximize constructive interference at the target location.

5.2.2 Experiment Apparatus

The basic optical setup is shown in **Fig. 5.1**, while different detectors and objectives are used in each experiment. Continuous-wave coherent laser light (532 nm, 5W) is projected onto an acousto-optic deflector (AOD, AODF 4090-7; G&H, Ilminster, UK) driven by a signal with frequencies from 60 to 100 MHz to generate multiple laser beams whose frequencies have been shifted. The radio frequency signals that drive the AODs are generated by an arbitrary function generator (AFG 3100; Tektronix, Oregon, US) followed by an RF power amplifier (325LA; Electronics & Innovation, US). A linear polarizer and half-wave plate are used to adjust polarization to match the input requirement of the AOD. The first-order frequency-shifted light from AOD focuses on the sample by a microscope objective (20X, NA 0.4). The light is recorded by an avalanche photodiode (APD430A2; Thorlabs, Newton, New Jersey, USA), and the signal from the detector is filtered and amplified before acquisition by a digitizer (M4I.4451-X8, Spectrum, Germany). For single frequency detection, we use two passive RF filters, DC block (BLK-222-75+; Mini-Circuits, USA) and low pass filter (DC to 1.7 MHz, EF510; Thorlabs, Newton, New Jersey, USA), to isolate the component around the desired frequency and then amplify the signal by a lock-in amplifier (SR844; SRS, USA). For all frequency detection, we use a preamplifier (DC-350 MHz, SR445A; Stanford Research Systems, Sunnyvale,

CA, USA). We use MATLAB to process the signal, calculate the optimized phase for each iteration, and generate a new waveform to drive the AOD through the AFG.

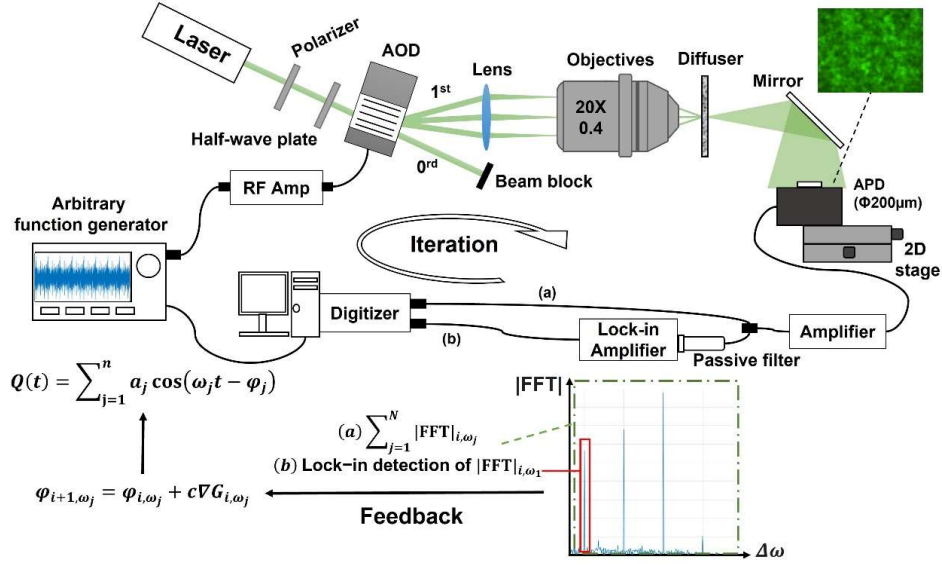


Fig. 5.1. Experimental setup of feedback-based wavefront shaping with lock-in detection of weak light.

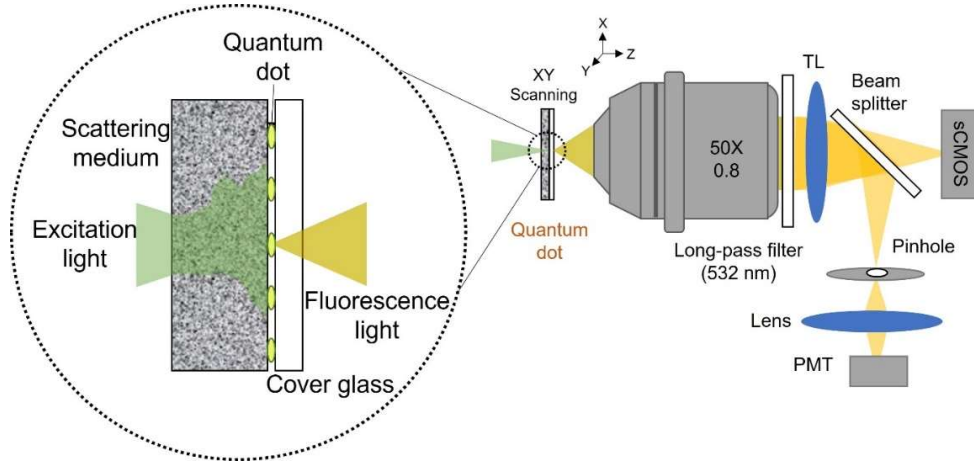


Fig. 5.2. Setup for fluorescence detection after transmission through a scattering media. Only the detection part is shown, and the rest is the same as in Fig. 5.1. The inset shows the sample configuration after zooming in.

5.3. Experimental Results

5.3.1 Spatiotemporal Focusing (Laser Speckles)

The transmission setup for wavefront shaping with laser light intensity as feedback is shown in **Fig. 5.1**. We use a diffuser (DG10-1500-MD, Thorlabs) as the scattering media. The signals are recorded by a silicon avalanche photodetector (APD, Thorlabs) with 400 MHz bandwidth. To optimize the performance of wavefront shaping, we match the speckle size with the APD sensor size (200 μm) by placing the APD 160 mm away from the diffuser. In this demonstration, 64 frequency components are generated by a function generator to drive the AOD to modulate the incident light. For the spatiotemporal focusing, we use a strategy similar to that in [18] in feedback-based wavefront shaping for spatiotemporal focusing, but no separate reference beam is involved. We use sample beams shifted by 60-100 MHz with the smallest spacing of 0.5 MHz for interference, so the beat frequency ranges from 0.5 to 40 MHz. The integration time is 30 ms for the lock-in measurements and spatiotemporal focusing experiment. In the spatiotemporal focusing experiment, the amplitudes of all beat frequency components can be significantly enhanced by using the summation of the amplitude from all beat frequency components as feedback and applying the gradient ascent method in feedback-based wavefront shaping. The lock-in amplifier, in this case, does not measure the signals. After the optimization, there is a temporal peak in the time domain signal due to the in-phase interference among all beat frequency components. The spatiotemporal focusing effect here is similar to the result in the reference article [18]. Because the phase has been updated instantly during feedback-based wavefront optimization, there is no need to recover the phases and refocus the beam. Since the period of the peak is

constant and determined by the envelope of the driving signals, we can apply digital time gating to isolate the signal with a short time window (~ 100 ns) around the peak. The effect of digital time gating is similar to using a pulsed laser for time gating [18]. Therefore, when the feedback is vital, the enhanced peak is very prominent in contrast to the background, in which all beat frequency components obtain substantial enhancement periodically in short durations (**Fig. 5.3(b-c)**), and the enhancement factor is comparable to the result obtained by the phase conjugation method using an AOD. In this case, it is feasible to perform 2D imaging of a cluster of quantum dots using the peak intensities derived from the digital time-gating of the time-domain signal (**Fig. 5.3(d-e)**). However, when the feedback is weak, it is difficult to observe any peak (**Fig. 5.4**). Because the sum of amplitudes of all beat frequency components is roughly equivalent to the instant intensity of the time-domain signal, this suggests that the performance of traditional feedback-based wavefront shaping, and potentially the phase conjugation method too, in case of weak signals, is not as good as the proposed method using lock-in detection of single beat-frequency components as the feedback.

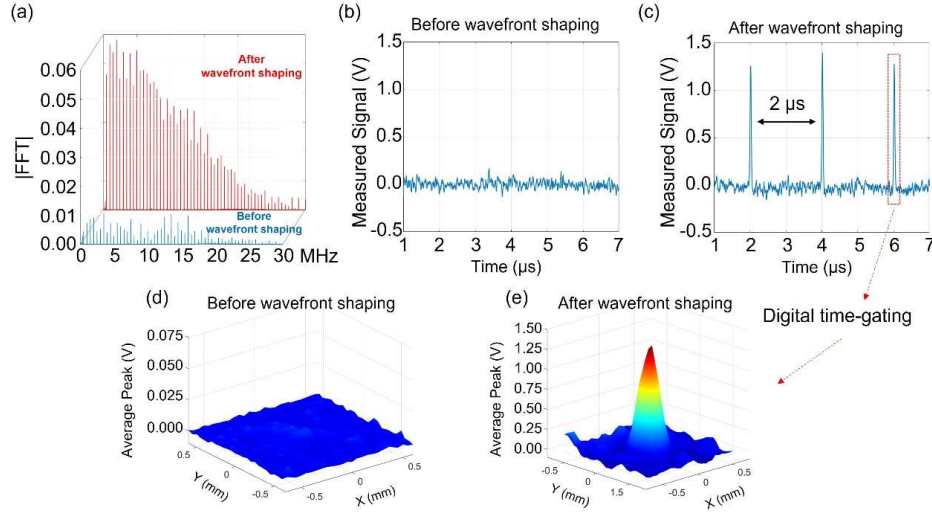


Fig. 5.3. Performance of wavefront shaping when using the sum of magnitudes of all beat-frequency components as feedback. (a) The magnitudes of all beat frequency components increase after wavefront shaping. (b-c) Temporal focusing is formed in the time-domain signal after wavefront shaping. (d-e) 2D imaging of a cluster of quantum dots using the peak intensities derived from the digital time-gating of the time-domain signal in (b-c). It can be seen that a clear image of the quantum dots is observed at the center in (e) after wavefront shaping, while there is only noise in (d) before wavefront shaping.

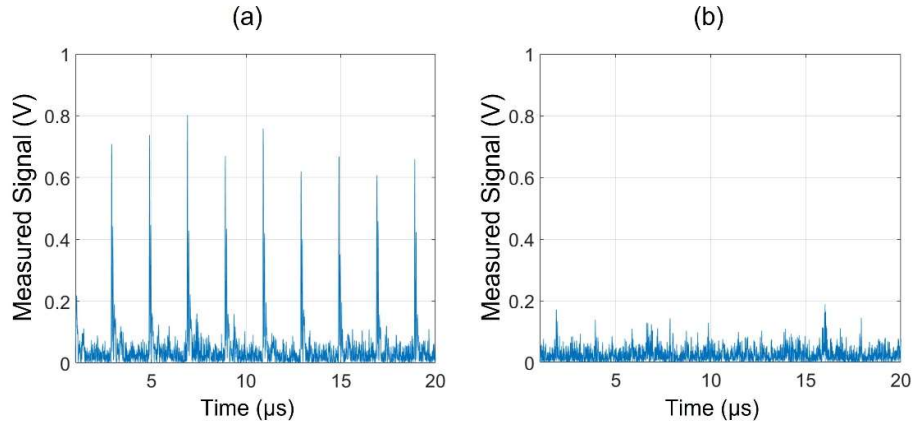


Fig. 5.4. Comparison of temporal focusing between strong and weak feedback after wavefront shaping. (a) Peaks formed by temporal focusing are prominent relative to the background for strong feedback, while only noise can be observed (b) when the feedback is weak.

5.3.2 Wavefront Shaping with Lock-in Detection for Laser Speckle in Transmission Setup

We explore using a passive filter and a lock-in amplifier for the weak laser light to enhance the SNR. In this method, we isolate and amplify the signal with one desired frequency and use its magnitude as the feedback for iterative optimization. The magnitude of the selected frequency component significantly increases after wavefront shaping, as shown in **Fig. 5.5(a-b)**. After optimization, we perform point scanning by mounting the APD on a motorized stage to generate a 2D image. The focal spot can be seen as shown in **Fig. 5.5(d)**. The enhancement factor is calculated from the 2D image after focusing on analyzing the performance. The ratio of peak intensity to the average background intensity values is defined as the enhancement factor [56]. Also, it is noted that the enhancement factor increases as the number of degrees of freedom goes up, as shown in Fig. 2(e).

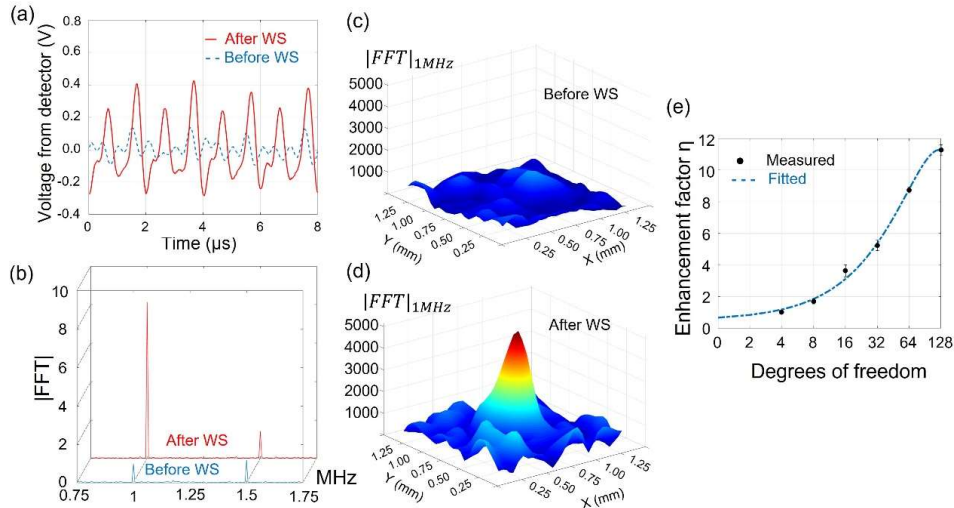


Fig. 5.5. Feedback in wavefront shaping in the (a) time domain and (b) frequency domain before and after wavefront shaping using AOD. Sample images (c) before and (d) after wavefront shaping generated by raster scanning the detector. (e) Enhancement factor as a function of the number of degrees of freedom. WS: Wavefront shaping.

5.3.3 Comparison in Enhancement Factor of Laser Light Transmitted through a Scattering Media among Different Feedback Strategies

After optimization using lock-in detection for the feedback at a single frequency, the magnitudes of all beat frequency components increase. This makes it possible to use the sum of magnitudes of all beat frequency components as feedback to create spatiotemporal focusing. Depending on light strength, there are different choices for feedback. For strong light, we could use the sum of amplitudes of all beat frequency components as the feedback to optimize the wavefront of the incident laser beam and generate spatiotemporal focusing [131]. Consequently, there is a total of three strategies for the selection of feedback to guide wavefront optimization, i.e.

Strategy 1. The magnitude of a single beat frequency component detected by a lock-in amplifier.

Strategy 2. The summation of magnitudes of all beat frequency components is calculated from the Fourier transform of the time-domain signal.

Strategy 3. Apply Strategy 1 in wavefront shaping first to achieve a decent initial signal, followed by applying Strategy 2 to achieve spatiotemporal focusing.

The enhancement factor is plotted as a function of detected optical power for feedback, as shown in **Fig. 5.6**, in which the laser light serving as the feedback was transmitted through a scattering media. A few points are worth noting in **Fig. 5.6**. The first point is that strategy 1, involving a single beat frequency and lock-

in amplification, yields a higher enhancement than strategy 2 when the detected signal's optical power is lower than 2.352 nW. Moreover, the opposite is seen when the detected signal is stronger. The higher signal is due to the SNR increase of the weak signal from using lock-in amplification in Strategy 1, which would yield more reliable feedback to wavefront optimization. Strategy 2 adds up the magnitudes of all frequency components, which is better than the traditional DC measurement but still does not work well for a weak signal.

When the detected signal's optical power is higher than 2.352 nW, the enhancement factor using Strategy 1 is lower than that using Strategy 2, as shown in **Fig. 5.6**. This finding indicates that Strategy 1 only optimizes wavefront for a single beat frequency component thus only yielding partial enhancement of the overall time-domain signal—in contrast, using Strategy 2 attempts to optimize the wavefront for the overall signal intensity, which is similar to the traditional choice of feedback, thus yielding higher overall enhancement. However, it can be seen from **Fig. 5.6** that Strategy 1 works for very weak optical signals, while Strategy 2 relies on a strong signal to start with. The combination of the two methods, i.e., Strategy 3, could extend the working optical power range of wavefront shaping to a lower limit while maintaining high enhancement factors.

While using a separate and strong reference beam for optical heterodyne detection as in the phase conjugation method [18, 19] can help increase the beat signal strength for coherent interference, it is limited when applied to *in vivo* measurements due to the safety threshold of laser irradiation or when highly sensitive detectors are required to capture weak beat signals because intense reference light could damage samples or detectors. In contrast, our method involving Strategy 1 does not require an intense reference beam because the

signal amplification step occurs inside the lock-in amplifier and thus is more suitable in such situations. In addition, optical heterodyne detection does not apply to the direct measurements of weak incoherent light, such as fluorescence or Raman signals.

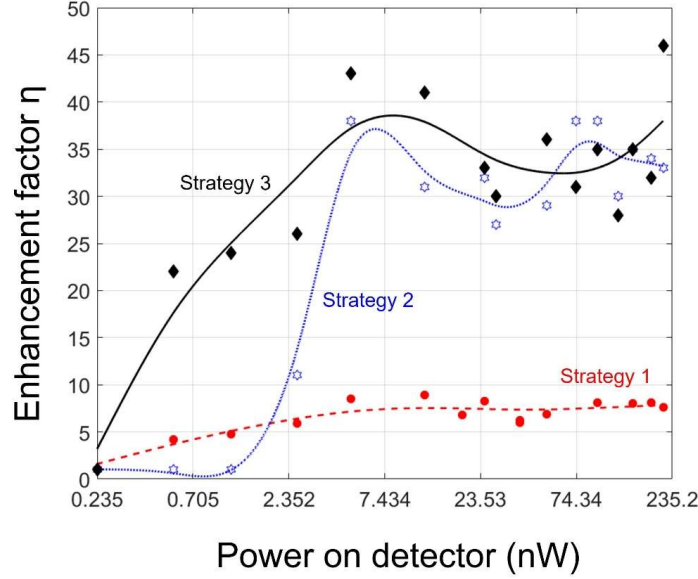


Fig. 5.6. Enhancement factor as a function of detected light power for three strategies different in the choice of feedback.

5.3.4 Fluorescence Enhancement in Transmission Setup

As an example of weak feedback, we further evaluate feedback-based wavefront shaping for fluorescence enhancement in a transmission setup (**Fig. 5.2**), in which quantum dots are used due to their excellent resistance to photobleaching compared to other fluorophores. Therefore, quantum dots were used for the fluorophore, and a series of TiO₂ phantoms (mixed with PDMS) served the scattering media sequentially. For much weaker fluorescence or Raman measurements, we use PMT as the detector instead of APD for higher sensitivity

to weak light. The integration times used in DC measurements and the lock-in detection of AC signals are both 30 ms. An sCMOS camera was used to locate a small cluster of quantum dots on a transparent coverslip, and a scattering media was then placed in front of the quantum dot to induce scattering. TiO_2 concentration and phantom thickness controlled the sample's optical thickness. The pinhole was scanned to image the fluorescence focal spot, similar to the earlier laser light measurements. Additionally, the sample was scanned for fluorescence imaging. Various images were formed by measuring the magnitude of the frequency component at 1 MHz or the pulse amplitude in temporal focusing, depending on the feedback strategy described in **section 5.3.3**. Each raw image was normalized by taking the ratio of each pixel value to the mean of representative background pixel values to facilitate comparison across images. Therefore, the resulting images in **Fig. 5.7** represent the contrast between foreground and background.

In the transmission setup where fluorescence measurements do not suffer from multiple scattering, it is observed that spatiotemporal focusing is only formed in the sample with a smaller optical thickness. In comparison, lock-in detection can enhance the signal in the sample with a larger optical thickness. The top row of **Fig. 5.7(b)** shows that for a thin sample ($L/l_t=2.1$), spatiotemporal focusing can be formed even using Strategy 2 without a lock-in amplifier. The contrast is better when using Strategy 3, which suggests that the hybrid strategy improves the outcome. In contrast, the bottom row of **Fig. 5.7(b)** shows that for a thick sample ($L/l_t=6.8$). Thus with weak feedback, Strategies 2 and 3 fail due to insufficient SNR for consistent wavefront optimization. Only Strategy 1 using the lock-in amplifier can yield a fluorescence image similar to the original one in

Fig. 5.7(a). It indicates that the lock-in detection method can increase the SNR and lower the limit of light power for effective feedback-based wavefront shaping. This observation highlights the power of the proposed method of beat frequency feedback with lock-in detection, i.e., Strategy 1, in case of weak feedback.

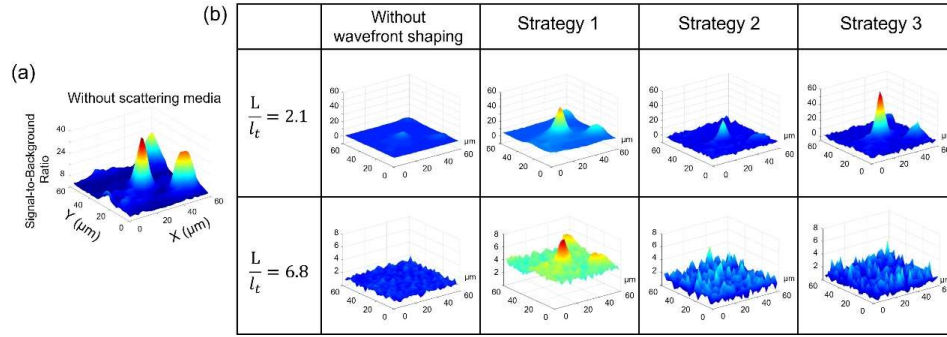


Fig. 5.7. 2D imaging of quantum dots clusters with different strategies. (a) Fluorescence imaging of quantum dot clusters without scattering media (b) fluorescence imaging after transmission through a scattering medium when using different wavefront shaping strategies in the choice of feedback. L : thickness; l_t : transport mean free path.

5.3.5 Fluorescence Enhancement in Reflection Setup

In a reflection setup shown in **Fig. 5.8(a)** to mimic the situation of *in vivo* measurements, all beat frequency components except the strongest one at 0.5 MHz are invisible in the presence of scattering media when fluorescence is affected by multiple scattering (**Fig. 5.9**). Hence, we can only use lock-in detection for fluorescence enhancement in the reflection setup subsequently. We use TiO_2 phantom (l_t : $\sim 97 \mu\text{m}$, sample thickness: $\sim 270 \mu\text{m}$) as the scattering medium. To evaluate the performance of wavefront shaping for fluorescence enhancement in the reflection setup, we investigate the fluorescence focal spot

(like the laser focus spot in **Fig 5.8(c-d)**) and fluorescence imaging by scanning the pinhole and the sample, respectively, after wavefront shaping. When scanning the sample, the scattering media and the fluorescent object are translated together. As shown in **Fig. 5.8(c, e)**, after one-time wavefront shaping in the region containing a quantum dot cluster isolated by the pinhole by using lock-in detection of the beat signal at 0.5 MHz as feedback, the contrast of the area corresponding to the pinhole position increases while no distinguishable focal spot is observed before wavefront shaping. The distinguishable focal spot after wavefront optimization shows that not only laser light but also fluorescence light can be used as feedback and enhanced after wavefront shaping using the lock-in detection method. With the same optimized wavefront, fluorescence images are generated by scanning the sample, showing that the overall contrast is also enhanced, and the reconstructed spatial information shown in **Fig. 5.8(f)** is similar to the original one obtained without scattering media shown in **Fig. 5.8(b)**. The contrast is very low without wavefront shaping, and some spatial features with weak fluorescence are missing, as labeled in **Fig. 5.8(d)**. Overall, the result shows that the wavefront shaping method with lock-in detection could directly recover the distribution of fluorophores through scattering media in a reflection setup.

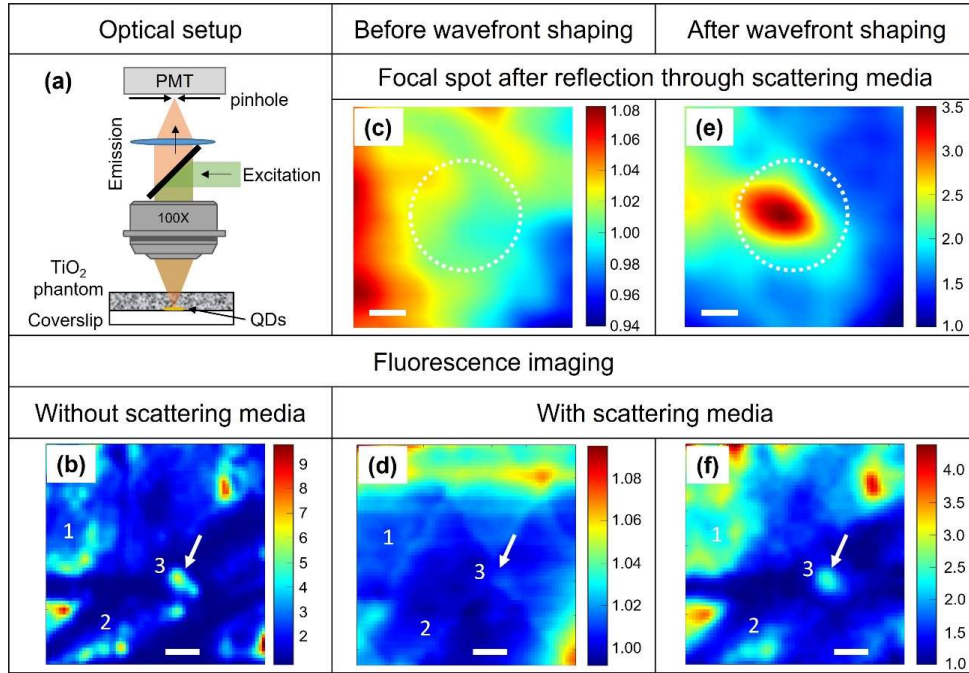


Fig. 5.8. Wavefront shaping with lock-in detection for fluorescence enhancement. The reflection setup is shown in (a). All raw images in (b-f) are normalized by taking the ratio of each pixel intensity to the mean of representative background pixel intensities. The focal spot images in (c, e) are generated by scanning the pinhole along with the PMT within an area of $6\ \mu\text{m} \times 6\ \mu\text{m}$ (corresponding to the sample plane), and fluorescence images in (b, d, f) are generated by scanning the sample within an area of $64\ \mu\text{m} \times 64\ \mu\text{m}$. The white circles in (c, e) and the arrows in (b, d, f) indicate the region from which fluorescence is measured for wavefront optimization. The labels (1,2,3) indicate the spatial features that are missing before wavefront shaping and can be recovered after wavefront shaping—scale Bar: $2\ \mu\text{m}$ (Focal spot), $10\ \mu\text{m}$ (Fluorescence imaging).

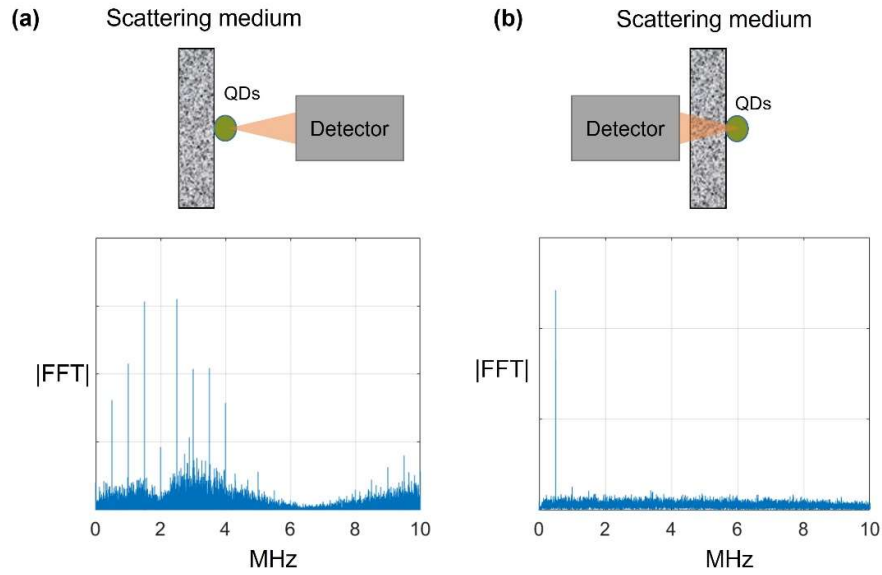


Fig. 5.9. Beat signals from fluorescence with or without multiple scattering. Multiple beat signals are to spot when fluorescence is not affected by the scattering medium, while only one or two peaks are distinguished from the background if the fluorescence suffers from the multiple scattering. (Integration time: 1 ms)

5.3.6 Raman Signal Enhancement in Reflection Setup

We apply wavefront shaping with lock-in detection to Raman spectral enhancement in a reflection setup shown in **Fig. 5.10**, in which titanium dioxide (TiO_2 , $1.5\ \mu\text{m}$) is used as the target and semitransparent tapes (Scotch Magic Tape 104) are used as the scattering medium (l_t : $\sim 250\ \mu\text{m}$, sample thickness: $\sim 250\ \mu\text{m}$). TiO_2 has two Raman peaks ($445\ \text{cm}^{-1}$ and $615\ \text{cm}^{-1}$), and semitransparent tape has no peaks within the region ($150\ \text{cm}^{-1}$ to $900\ \text{cm}^{-1}$). Note that the tape does not emit Raman signals in the target region, according to our test. We use a monochromator (CS260, Cornerstone; Newport) to measure the whole Raman spectra by scanning the wavelength. With the same monochromator for wavefront shaping, we isolate the certain Raman peak ($445\ \text{cm}^{-1}$) of TiO_2 and use the signal as the feedback for the wavefront shaping (**Fig. 5.10**).

To measure the data point at each Raman shift in the Raman spectra, the emitted light is detected by a PMT, and a lock-in amplifier measures the signal at the desired beat frequency ($0.5\ \text{MHz}$ in the experiment). The amplitude (output) of the signal at the desired frequency ($0.5\ \text{MHz}$) from the lock-in amplifier is used as the reading at each wavelength. The integration time is set to $30\ \text{ms}$ at every point. After scanning across the selected wavelength range, each wavelength set by the monochromator was converted to Raman shift serving as the x value, and the corresponding reading from the lock-in amplifier was used as the y value. The Raman spectrum of the same sample measured by a commercial spectrometer served as a reference for comparison to ensure that the measured Raman spectra of TiO_2 were correct.

As shown in **Fig. 5.11** and **Fig 5.12**, the performance of wavefront shaping on Raman scattering enhancement by using AC (the magnitude of the beat frequency component at 0.5 MHz) or DC (the average intensity in the time domain) signals as feedback. Fig. 5.11(b-f) compares Raman spectra before and after wavefront shaping from 150 cm^{-1} to 900 cm^{-1} where TiO_2 has the characteristic Raman speak (445 and 615 cm^{-1}). These peaks are submerged in the background without optimization (**Fig. 5.11(c-d)**). After wavefront shaping with lock-in detection of the magnitude of 0.5-MHz beat frequency signal, the contrast of Raman peaks of TiO_2 relative to the background increases when using the 0.5-MHz beat frequency magnitude for spectral reconstruction (**Fig.5.11(f)**). Two major peaks become distinguishable from the background, and the entire spectral shape agreed nicely with the spectrum measured without scattering media (**Fig. 5.11(b)**). In contrast, using the DC signal for spectral reconstruction does not yield any meaningful spectral features (**Fig. 5.11(e)**) after wavefront shaping. In addition, when using the DC signal as feedback, the spectral shape is not significantly improved before and after wavefront shaping (**Fig. 5.12**).

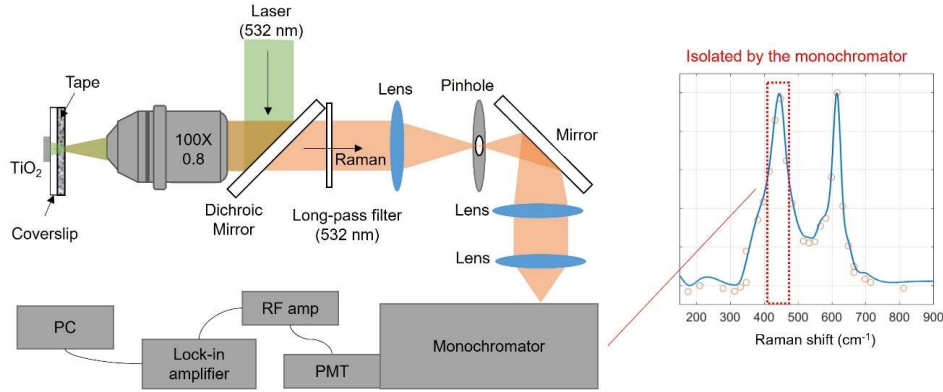


Fig. 5.10. Optical setup (detection part) for Raman scattering enhancement. The monochromator was used to isolate the specific Raman peak as the feedback for the wavefront shaping. The monochromator was also used to generate the Raman spectrum within the region (150 cm^{-1} to 900 cm^{-1}) where TiO_2 has the characteristics peaks (445 and 615 cm^{-1}).

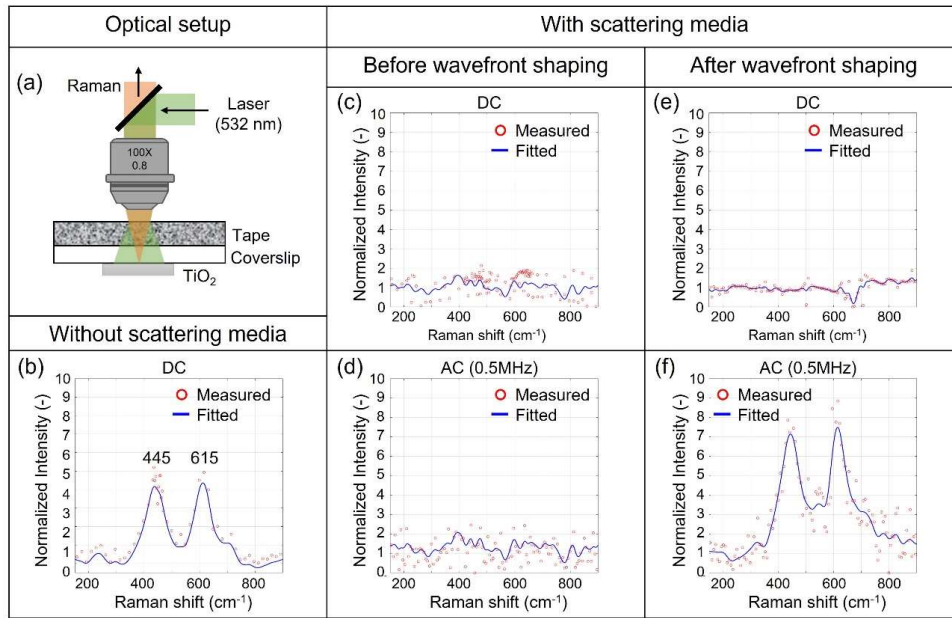


Fig. 5.11. Wavefront shaping with lock-in detection for Raman scattering enhancement. The reflection setup is shown in (a). All spectra (b-f) are normalized by their maximum intensity. (b) The Raman spectrum measured without scattering media shows two Raman peaks of TiO_2 . With scattering media, the spectrum is dominated by noise, and no peaks are distinguishable from the background, as shown in (c-d) before wavefront shaping. After using AC feedback for wavefront shaping, the magnitude of the AC signal (f) reveals two peaks that can be attributed to TiO_2 , while the spectrum reconstructed by the DC signal, as shown in (e), remains featureless.

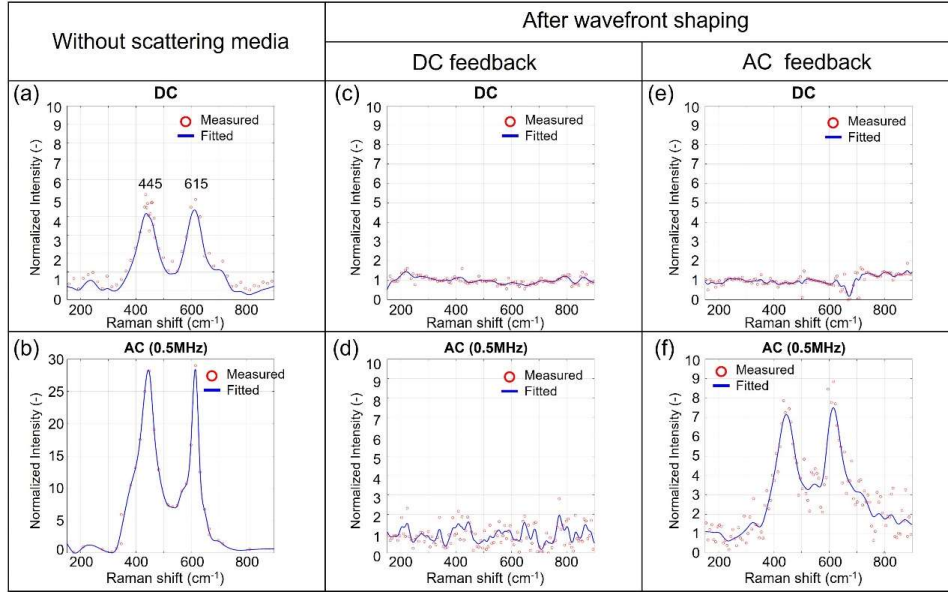


Fig. 5.12. Comparison of feedback wavefront shaping using DC or AC signals as the feedback. The title of each subfigure gives the quantity used for spectral reconstruction, in which “DC” and “AC (0.5 MHz)” mean the average intensity in the time domain and the magnitude of 0.5 MHz beat frequency in the frequency domain, respectively. (a-b) The Raman spectra of the TiO_2 measured by the monochromator show that the TiO_2 has two prominent peaks (445 cm^{-1} and 615 cm^{-1}). (c-d) After using the DC signal as feedback for wavefront shaping, the two peaks are still buried in the background and noise, while (f) using the AC signal could significantly increase the signal-to-background ratio that the two peaks are distinguishable from the background and the spectrum becomes similar to the (a-b) original one without scattering media. However, the DC signal still is noise-dominated, as shown in (e) after wavefront shaping with AC signals as feedback.

5.4. Discussion

5.4.1 Comparison of Spatiotemporal Focusing and Lock-in Detection

Compared to spatiotemporal focusing, which involves the summation of all frequency components as feedback, the enhancement factor obtained using a single frequency component for optimization is relatively low (**Fig. 5.6**). The advantage of using lock-in detection of only one beat frequency is that this approach works for much weaker light due to the ability of the lock-in amplifier in weak signal detection. It is feasible to perform wavefront shaping using a hybrid approach to achieve a high enhancement factor for weak signals, in which the lock-in detection of a single beat frequency component is used first to achieve a decent initial signal then, followed by applying the summation of all frequency components to achieve spatiotemporal focusing.

When using the sum of amplitudes from all beat signals as the feedback in the wavefront shaping, it is feasible to form spatiotemporal focusing with a good enhancement factor ($\eta \sim 35$) as shown in our study (**Fig. 5.6**), which is much closer to the theoretical value ($\eta \sim 50$) than the approach of lock-in detection for 64 degrees of freedoms as used. It is worth noting that we only use the amplitude of a single beat signal in the lock-in detection approach, which is only a small portion of all beat signals and thus may suffer from lower sensitivity to wavefront shaping. In this case, the enhancement factor is significantly lower ($\eta \sim 8$). In short, the enhancement factor calculated for spatiotemporal focusing formed by the time domain signal is comparable with the theoretical **equation (5.1)**. We have not been able to derive or find an equation to predict the enhancement factor for the lock-in approach using a beat signal with a certain radiofrequency.

The enhancement factor is much higher in spatiotemporal focusing than in spatial-only focusing. The mathematical simulation implemented in MATLAB indicates that the enhancement factor could be low even after applying the spatiotemporal focusing approach if we only calculate the enhancement factor from the magnitude in the frequency domain (**Fig. 5.13(c)**). The low enhancement factor is similar to the result of the lock-in method (**Fig. 5.13(b, d)**). The signal isolated from the time-domain signal provides the spatiotemporal focusing information, while the amplitude of the beat signal only provides the spatial information. Hence, the highest enhancement factor only can be retrieved from the time-gating signal after spatiotemporal focusing (**Fig. 5.13(a)**).

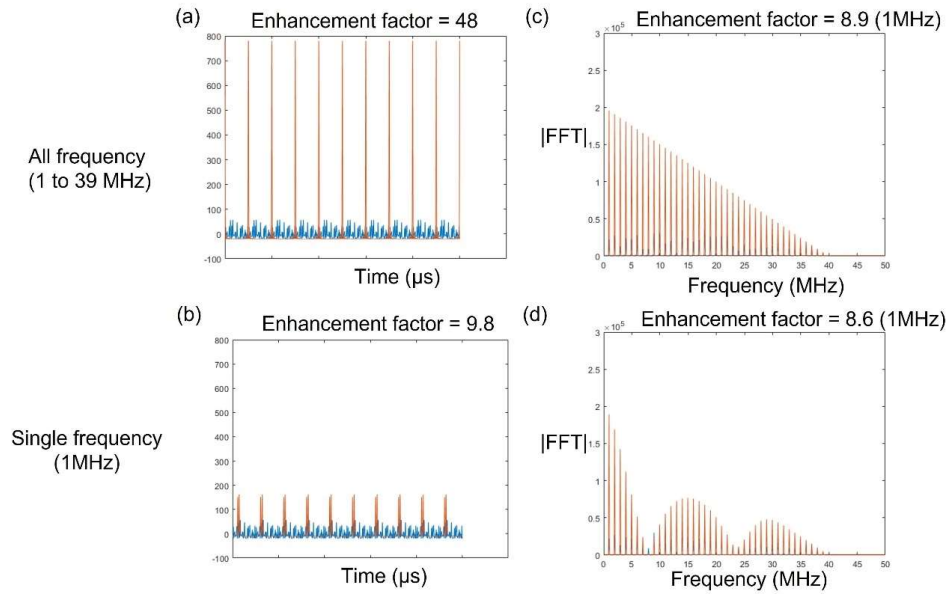


Fig. 5. 13. MATLAB simulation.

5.4.2 Memory Effect

The sample's high scattering coefficient could cause memory effect loss during scanning. In the experiment of fluorescence enhancement, the memory effect works well in this study for the following reasons.

The first is that the phantoms are relatively thin in our setup. The scattering media used in the experiment for **Fig. 3** is a TiO_2 phantom (l_t : $\sim 97 \mu\text{m}$, sample thickness: $\sim 270 \mu\text{m}$). The sample has a thickness of 2.7 transport mean free path with the parameters. Compared to those samples as thick as over ten transport mean free path [10], the sample here is relatively thin. The article [10] reports that with rotated incident light, even passing high scattering media (with a thickness of $5.1 \pm 1.1 \mu\text{m}$ and transport mean free path of $0.75 \pm 0.15 \mu\text{m}$), it is still possible to scan the sample for 2D imaging in a small region due to the memory effect. In our case, we do not rotate the incident beam to scan the sample. Instead, we translate the sample for 2D imaging. The optimized wavefront is partially effective in the region outside the optimization area.

The nature of the phantom could be another reason for the extended memory effect. We mixed the TiO_2 into PDMS and made it a solid phantom that was as homogenous as possible. The homogeneity could increase the similarity of scatterer distribution among different positions and enlarge the applicable region of the memory effect.

5.4.3 Improvement of Raman Signal Measurement

In the current setup for Raman signal enhancement, we use a monochromator to isolate the Raman peak embedded with a specific beat frequency and use it as feedback for wavefront shaping. However, the efficiency of the monochromator is relatively low, and energy loss in the detection part wastes the light. In the experiment, to avoid uncertain factors and fairly investigate the performance of wavefront shaping, we used the same setup with the same photodetector for DC and AC measurement for comparison instead of using a commercial spectrometer for DC measurement. To further improve the detection, we can use a DMD spectrometer to isolate multiple Raman peaks as the feedback signal to increase the signal. Also, compared to the CCD with a cooling system that can be down to -80°C , the current PMT we are using does not have a cooling system that could cause thermal noise.

5.5. Conclusion

In summary, the above findings suggest that the lock-in beat frequency detection method developed in this study is particularly suitable for measuring weak signals, such as fluorescence and Raman scattering in a reflection setup. Feedback wavefront shaping with a beat signal generated by AOD and lock-in detection can isolate and enhance the weak target signal buried in a noisy background. The technique could be potentially used for *in vivo* measurements that cannot be measured in a transmission setup. Because of the fast response of the AOD, this technique could be made much faster to achieve real-time measurements once integrated with electronics for the instant generation of driving radio frequency signals for AOD.

Chapter 6

Accelerating Feedback-Based Wavefront Shaping with FPGA Control and AOD Modulation

6.1 Introduction

In biological samples, most energy of a light beam focused by an objective is dissipated due to multiple scattering beyond one transport mean free path [132], which diminishes the performance of optical techniques. Wavefront shaping [40] is demonstrated to be capable of getting around multiple scattering by modulating the property of incident light to control scattered light and form highly constructive interference in a target region. Wavefront shaping attempts to find the optimized phase and amplitude distributions of incident light to reduce or remove multiple scattering artifacts, and the resulting optimized wavefront is usually only applicable to a particular scattering medium. The wavefront shaping technique has been demonstrated to work well for static samples such as a diffuser, a TiO₂ phantom, a semi-transparent tape, and a tissue slice in many published articles and this dissertation (**Chapter 3** to **Chapter 5**). However, for non-static samples, the motion of light scatterers inside causes changes in the distribution of the refractive index and, in turn, the scattering field. The correlation between the initial speckle pattern and the new speckle pattern becomes small at the next time point. Since each wavefront is optimized for

specific speckle fields, it will lose its capability of effectively focusing at the next time point if not updated in time in dynamic samples. For example, a live biological sample often has a short speckle correlation time, in which the speckle field changes rapidly due to breathing and blood flow [49], in which case slow wavefront shaping is ineffective. The refresh rate of wavefront optimization is the limiting factor in this situation.

To address the issue, high-speed wavefront shaping is required to catch up with the short correlation time. Digital optical phase conjugation (DOPC) [49] has been proposed to perform snapshot wavefront shaping that can focus on a sample within a millisecond scale, including time-reversed ultrasonically encoded light (TRUE) [70] and time reversal of variance-encoded light (TROVE) techniques [71]. However, these techniques are not able to work directly for incoherent light measurements like fluorescence, which represent essential applications of wavefront shaping [45]. Iterative wavefront shaping [38], on the other hand, can be used for incoherent light measurements, as demonstrated earlier [45, 133]. Although wavefront shaping cannot directly modify the resulting incoherent light, the incoherent light intensity can be used as feedback to optimize the wavefront of incident coherent light. However, the iterative procedure slows down the whole optimization, making it challenging to apply to a sample with a short correlation time. To overcome the limit, microelectromechanical systems (MEMS) spatial light modulators (SLMs) [53], grating light valves (GLVs) [54], and digital micromirror devices (DMDs) [65, 134] have been proposed to speed up the iterative optimization for its higher refresh rate. Among the techniques, an acousto-optic deflector (AOD) is advantageous compared to the above devices because of the higher speed. The

acousto-optic effect of AOD promises a short refresh time ($< 1 \mu\text{s}$) [1] for beam shaping without moving a component to avoid mechanical artifacts.

Light can be manipulated spatially and temporally by wavefront shaping to form a sharp focus behind scattering media for continuous-wave or pulsed laser sources [40, 135]. Compressing light in the time domain, as in temporal focusing, can significantly increase the signal contrast at a target location compared to spatial focusing only. For instance, compressing light can generate an ultrashort laser pulse in multi-photon microscopy [131] to enhance the excitation energy and resolution. Temporal focusing has been demonstrated to focus laser light behind a diffuser by using AOD with DOPC in milliseconds [75]. However, this and most other methods in the literature for high-speed wavefront shaping can only enhance laser light but not incoherent light. Enhancing incoherent light directly without using coherent light parameters as intermediate steps is essential in extending wavefront shaping to biomedical applications in which fluorescence spectroscopy/imaging is broadly adopted. Therefore, it is necessary to develop high-speed wavefront shaping to enhance incoherent light, such as fluorescence light signals directly.

In this study, we demonstrated that feedback-based wavefront shaping based on a single acousto-optic deflector (AOD) with FPGA acceleration could speed up signal enhancement with a continuous wave laser source. With the digital time-gating isolation of the feedback signal, spatiotemporal focusing with high contrast for laser light can be formed behind dynamic scattering media in milliseconds with sufficient enhancement. Moreover, this technique enhances fluorescence directly behind dynamic samples with a short correlation time. To our best knowledge, there have been no similar reports in the literature in which

feedback-based wavefront shaping with AOD is used to enhance incoherent light from dynamic samples directly.

6.2 Principle and Experimental Apparatus

6.2.1 Principle of FPGA-Based Wavefront Shaping

In chapter 3, we used a computer to change the phase of an SLM and an sCMOS camera or spectrometer to detect the signal from samples. In the lock-in detection method of beat frequency components, as in **Chapter 4**, we used a computer to program the output of a function generator to an AOD and a digitizer to record the signal from a photodetector. With the above methods, data transfer among different devices is time-consuming. For instance, refreshing an arbitrary waveform generated by a computer and sending it to a function generator takes longer than 100 ms. The latency makes wavefront shaping slow, especially for iterative optimization. To reduce the time of data transfer among devices and fully utilize the high speed of the AOD and photodetector, all signal processing steps are performed in an FPGA (VC707, Xilinx) for high-speed wavefront shaping in this study to catch up with the short correlation time of a dynamic sample. In addition, the highly parallel computing ability of FPGA can significantly speed up data processing and signal generation. Generally, the signal processing procedure can be categorized into two parts (1) waveform generation and (2) feedback signal analysis for optimized phase calculation. The two parts will be introduced below:

- *Real-time Waveform Generation*

To send an RF signal to drive the AOD, we use FPGA to generate a digital signal first with multiple RF components and then convert it to an analog signal with a

digital-to-analog converter (DAC; AD9144; Analog Devices). To generate a waveform with a stable phase, we mathematically calculate the waveform using a common wave equation, as shown in equation (6.1).

$$S(t) = \sum_i^{i=N} A_i \cos(2\pi f_i t + \phi_i) \quad \text{Eq. (6.1)}$$

where A_i is the amplitude, f_i is the frequency value and ϕ_i is the phase of the i -th RF component, and N is the number of different frequencies. Only phase modulation is used in the current experiment; therefore, the amplitude (A_i) is always 1. In FPGA, we create a lookup table (LUT) for the cosine function, which can be used to calculate the cosine function efficiently. To further reduce the required computation, we use integer values from 0 to 8000 (the FPGA's memory size limits this maximum number) to represent 0 to 2π , equivalent to assigning an integer index number to every discrete angle between 0 and 2π . The procedure of waveform generation is simplified as follows:

$$(\text{Step 1}) \ 2\pi f_i t = 32 \times f_i \quad \text{Eq. (6.2)}$$

$$(\text{Step 2}) \ 2\pi f_i t + \phi_i = \phi_i + 2\pi f_i t \quad \text{Eq. (6.3)}$$

$$(\text{Step 3}) \ \cos(2\pi f_i t + \phi_i) = \text{LUT}(2\pi f_i t + \phi_i) \quad \text{Eq. (6.4)}$$

$$(\text{Step 4}) \ S(t) = \sum_i^{i=N} \cos(2\pi f_i t + \phi_i) \quad \text{Eq. (6.5)}$$

Each equation is elaborated on below.

(1): The clock frequency of the DAC card is 250 MHz, which corresponds to a time increment (Δt) of 4 ns. Therefore, the frequency increment becomes $2\pi \cdot \Delta t \rightarrow 8000 \times 4 \text{ ns} = 32000 \text{ ns}$. Since the driving frequency f_i has a unit of MHz ($10^{-6}/\text{s}$), $2\pi f_i \cdot \Delta t$ becomes $32f_i$.

- (2): Calculate the overall phase by adding the contributions of angular frequency and phase delay terms.
- (3): LUT represents “Look-up Table.” We can avoid calculating the cosine function with a lookup table and directly get the result. EXCEL or MATLAB can generate the lookup table, and numbers need to be rounded up to integers between 0 to 8000.
- (4): The sum of calculated values for different frequencies becomes one point of the waveform.

Due to parallel operation in FPGA, the calculation for all frequencies can be implemented simultaneously. Therefore, we can update the waveform with a 32 ns delay. Unlike a function generator, which uses the playback function to repeat the waveform from memory, FPGA keeps generating new points constantly. The customized waveform generator makes the FPGA-DAC useful for accommodating special requirements. The performance of RF signal generation is affected by the LUT size and the performance of the DAC card. Although the FPGA-based waveform generator is less precise than a commercial function generator, its performance is sufficient for the study.

- *Analysis of Feedback Signal for Optimizing Wavefront to Generate Temporal Focusing*

Because an AOD works as a dynamic grating (refer to **Chapter 4**), the phase of light passing an AOD can be treated as a constant only within nanoseconds. Due to the excellent repeatability of the waveform, the phase of light at the exact moment in each waveform cycle is precisely identical. At this frozen moment, phase modulation in an AOD is similar to that in an SLM for feedback-based

wavefront shaping (**Chapter 3**), which targets the signal corresponding to the specific timing of the waveform. It thus can be utilized for maximizing feedback intensity and refocusing light. Because the optimized phase only works for nanoseconds, temporal focusing can be generated, as shown in **Fig 6.1**. To perform wavefront shaping, the amplified analog signal from an avalanche photodetector (APD; APD430A, 400 MHz; Thorlabs) is converted to a digital signal by an analog-to-digital converter (ADC, 1 GHz; AD9680; Analog Devices) with high bandwidth. The signal within a 60-ns time gate is extracted digitally and used as feedback during iterations. The optimization maximizes the signal within the time gate to generate a peak in the time domain. To test the performance of spatiotemporal focusing, we use this method to focus light through a diffuser (DG10-1500-MD; Thorlabs) and scan the APD to measure the 1-D profile (**Fig. 6.2(A)**). As shown in **Fig. 6.2(B-C)**, the focal spot is formed with a width of 240 μm , close to the APD sensor diameter (200 μm) after wavefront shaping.

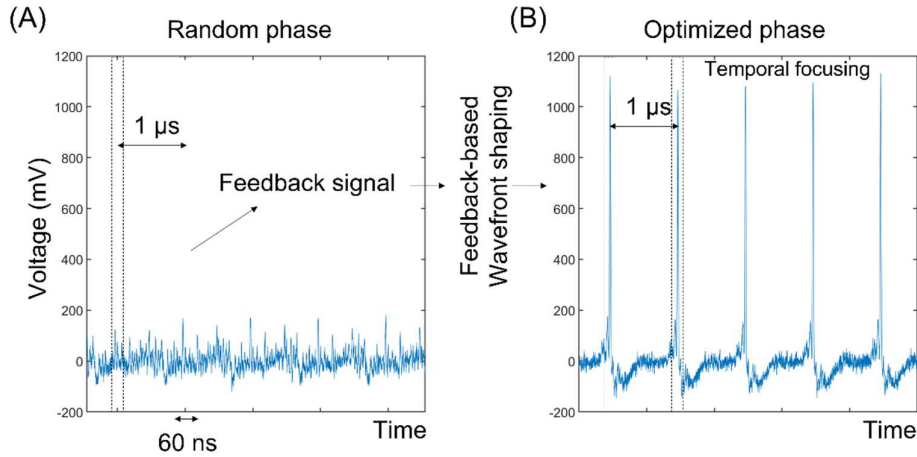


Fig. 6.1. Temporal focusing generated by AOD modulation(A) With a fast photodetector, the signal within 60 ns can be isolated and serve as feedback for wavefront shaping. (B) After wavefront shaping, the signal within the time gate can be significantly enhanced to form temporal focusing. (Degrees of freedom: 32).

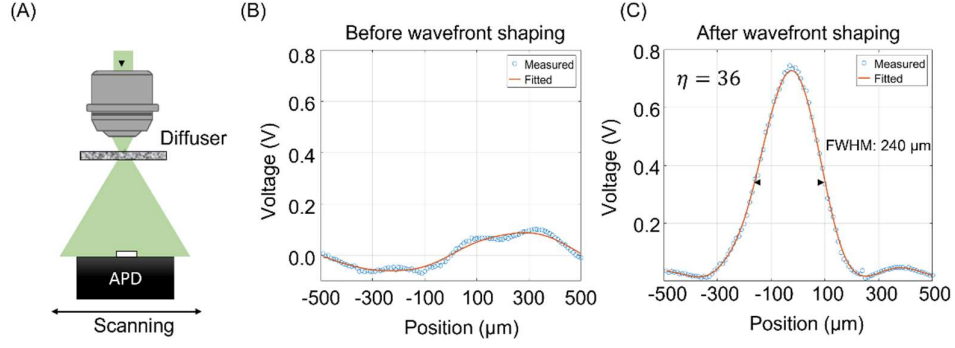


Fig. 6.2. Focal spot is formed behind a diffuser after wavefront shaping. (A) To observe the focusing performance behind a diffuser, we laterally scan the APD to generate a 1D profile (B) before and (C) after wavefront shaping. The data is obtained from the isolated signal within the time gate shown in (Fig.6.1(B)). In (C), a clear focal spot is generated after wavefront shaping with an FWHM of 240 μm close to the sensor diameter (200 μm). (Degrees of freedom: 32)

- *Workflow of Feedback-based Wavefront Shaping with FPGA Acceleration*

To avoid data transfer delays among devices, we use a closed-loop setup in which all optimization processes are implemented in the FPGA board. An example of the time chart for wavefront shaping with 32 degrees of freedom is shown in **Fig. 6.3**. Generally, there are three steps in the procedure to update the phase (φ_i) in the gradient ascent algorithm (**Equation 6.6**) (refer to **section 2.4.2** in **Chapter 2** for details):

$$\varphi_{i+1} = \varphi_i + c \nabla G_i \quad \text{Eq. (6.6)}$$

(1) Measure the gradient value (∇G_i):

In the first step of the algorithm, we need to measure the gradient at each driving frequency. During the gradient measurement, the ADC module connected to the FPGA board sequentially retrieves the filtered signal from the photodetector after amplification.

(2) Find the optimal step size (c) using the golden search method:

The gradient and step size determine the optimized phase in the gradient ascent algorithm. Therefore, after the gradient measurement is completed, the golden search method is used to find the optimal step size by finding a convergent solution.

(3) Update the phase φ_i with φ_{i+1} and generate a new waveform:

After the step size is determined, the new waveform is generated according to the optimized phase. However, the optimized phase could be ineffective for several reasons, such as noise influence. Therefore, we compare the feedback signal for the new and initial phases to determine the final optimized phase for a stronger signal. After each iteration is completed, a waveform with the final optimized phase will be generated to serve as the new driving signal for AOD.

The processing time for each iteration is related to the measurement time and the degrees of freedom. Depending on signal strength, we can use a longer measurement time to increase the signal-to-noise ratio or a shorter measurement time to decrease the time of each iteration. In the current setup, the time for each iteration in wavefront optimization can be estimated as follows:

$$t_{ws} = t \times [(N + 1) + 3 \cdot G] \quad \text{Eq. (6.6)}$$

where t_{ws} is the time spent on each iteration, t is the measurement time of the feedback signal at each RF, N is the degrees of freedom (i.e., the number of RFs involved), and G is the number of rounds when performing the golden search method. For instance, when N , i.e., the DOF, is 32, and G is 15, the time for each iteration will be $16 \times [(32 + 1) + 3 \times 15] = 1248 \mu s$.

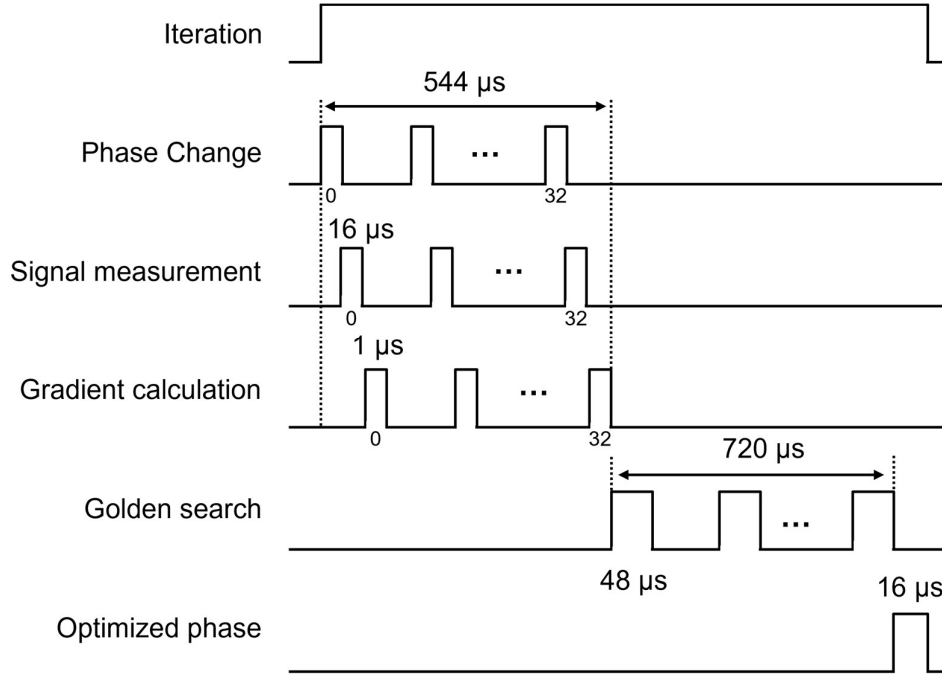


Fig. 6.3. Time chart of feedback-based wavefront shaping using FPGA with 32 degrees of freedom. In general, there are three steps: (1) Calculate the gradient values for all driving frequencies with sequential measurements. (2) Use the golden search method to find the step for the formula to calculate the new phase. (3) Determine the optimized phase and generate a new waveform.

6.2.2 Experimental Setup

The basic setup for FPGA-based wavefront shaping is shown in **Fig. 6.4**. Because the refresh rate of the AOD is affected by the beam size of the incident beam, we manage to minimize beam size by focusing light near the AOD to decrease the time for the entire light beam to be effectively modulated. Therefore, we use a lens with a long focal length ($f = 500 \text{ mm}$) with a large f-number to focus light and adjust the beam waist to a rational size without damaging the AOD and maintaining the beam shape of the first-order diffracted light. For this purpose, the frequency-shifted light will be focused on a sample by a 20X objective in this study. In transmission measurements, we put an APD under the sample at a certain distance to measure laser speckles where the speckle size matches the

diameter of the APD sensor. The signal from the APD will be amplified by a radiofrequency (RF) amplifier and then converted to a digital signal by the analog-to-digital converter (ADC) module in the FPGA unit for signal processing. After the optimized phase is calculated, the new waveform will be sent to the AOD from the DAC module in the FPGA unit. When fluorescence is measured, a slightly different setup will be used, as illustrated in the dashed rectangle on the right side of **Fig. 6.4**. A long-pass filter will be used to reject excitation light. Emitted fluorescence will be focused onto a pinhole, and a PMT will detect the spatially filtered signal.

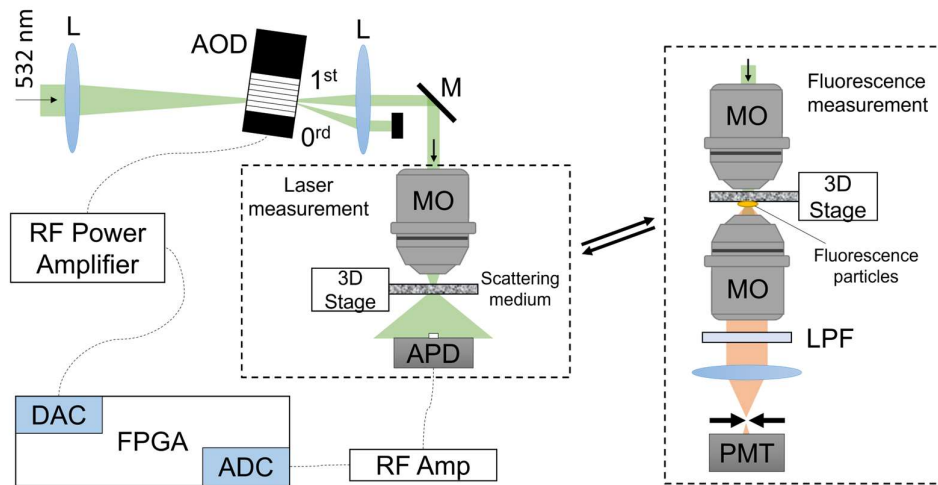


Fig. 6.4. Schematic of the setup for FPGA-based wavefront shaping for laser speckle and fluorescence measurement. AOD: Acousto optic deflector; L: Lens; FPGA: Field-programmable gate array; DAC: Digital-to-analog converter; ADC: Analog-to-digital converter; MO: Microscope objective; M: Mirror; APD: Avalanche photodiode; RF Amp: Radiofrequency amplifier. LPF: Long-pass filter (532 nm); PMT: Photomultiplier tube.

6.2.3 Dynamic Samples

In this study, two kinds of dynamic samples are prepared.

- *Static Samples on a Motorized Stage*

The motion of a static sample changes the scattering pattern of the illumination field. By controlling the velocity of the motorized stage, the varying speed of the scattering pattern can be adjusted. Also, the motorized stage can control the moment the motion starts. Because the motion caused by the motorized is for translating the whole sample with a constant speed, this type of sample acts to simulate the influence of breathing on tissue measurements.

- *Intralipid-Gelatin Phantoms [54, 70]*

The procedure makes the phantom: gelatin (G9391; Sigma-Aldrich) is first dissolved in water at 40 degrees, then intralipid (I141, 20%; Sigma-Aldrich) is mixed in the solution. The phantom is put into a fridge to cool down and form a gel for further use. In this phantom, intralipid particles generate multiple scattering of light, and the gelatin content controls the viscosity of the solution. The Brownian motion of intralipid particles causes a change in the scattering pattern over time. The viscosity, determined by the gelatin concentration, affects the diffusion rate of the intralipid particles. This kind of sample acts to simulate the influence of body fluid motion on tissue measurements.

The crucial difference between dynamic and static samples is the correlation time. The speckle correlation time of a dynamic sample is much shorter than a static sample. The speckle pattern of a static sample is almost unchanged for a long time if it is fixed well. The correlation time can be

calculated by the decay of the correlation coefficient with the following formula [5]

$$I = A \cdot \exp\left(-2t^2/\tau^2\right) + c \quad \text{Eq. (6.7)}$$

where A is the correlation factor, c is a constant, t is time, and τ is the correlation time. The correlation time is when the intensity is around $1/e^2$ (~13%) of the initial value. Therefore, we can take time-lapse 2D images and use MATLAB's 2D correlation coefficient function to retrieve the coefficient and generate a decayed curve, as shown in **Fig. 6.5**. The correlation time can be estimated by fitting the data to **equation (6.7)**. The method can be used to estimate the correlation time of both motorized static samples and intralipid-gelatin phantoms.

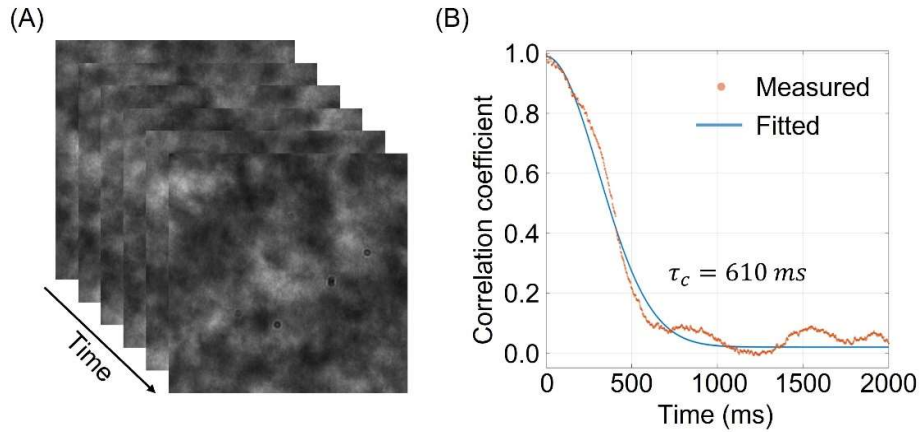


Fig. 6.5. Correlation time determination of a dynamic sample. (A) A Series of 2D speckle images taken at different times are used to calculate the correlation coefficient over time. (B) The correlation coefficient is calculated between every subsequent image and the first image. The data are fitted to equation (6.7) to find the correlation time.

6.3 Experimental Results

6.3.1 Continuous Wavefront Shaping vs. Single-time

Wavefront Shaping

The performance of iterative wavefront shaping is affected by noise and signal drift in feedback. For static samples, the result of continuous wavefront shaping on static samples shows a stabilized intensity with minor fluctuation after achieving the maximum (**Fig. 6.6(A)**). In contrast, the motion of a dynamic sample or the movement of scatterers inside can change the speckle pattern, which affects the measured signal used for the iterative algorithm. Therefore, the intensity variation during continuous wavefront shaping is very prominent (**Fig. 6.6(B)**). Even though the fluctuation is due to the dynamic motion, the signal can still be maintained at a high level with continuous wavefront shaping while the intensity drops over time if using one-time wavefront shaping.

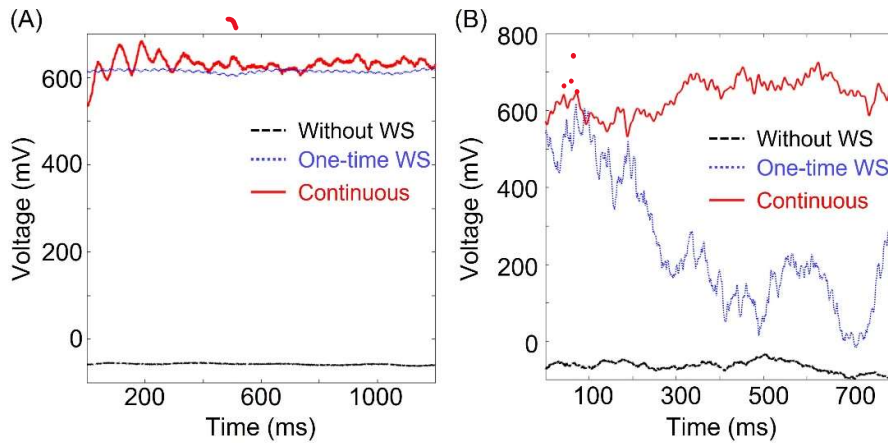


Fig. 6.6. Effect of continuous wavefront shaping on a dynamic sample. (A) For a static sample (Diffuser), the scattering pattern does not change with time, so an optimized wavefront obtained at the one-time point can keep the signal at a high level within a period. (B) The signal will drop after wavefront shaping for a dynamic sample (Intralipid-gelatin phantom) since the scattering pattern keeps changing. With continuous wavefront shaping, the signal can be maintained at a high level. WS: Wavefront shaping.

6.3.2 Continuous Wavefront Shaping on Dynamic

Samples for Laser Speckle Enhancement

- *Samples Moving on a Motorized Stage*

To test the performance of continuous wavefront shaping, we mount a diffuser on a 1D motorized stage. The motion induced by the motorized stage will change the speckle field on the illuminated area, causing the failure of initial wavefront shaping, and the rate of change depends on the speed of the motorized stage. The correlation time can be evaluated as described in **section 6.2.3**. In this experiment, we first optimize the signal to its maximum and then trigger the motorized stage to move the sample. As shown in **Fig 6.7(A)**, the signal drops to a very low level because the speckle field becomes decorrelated with the initial pattern. So, the initial location A and the final location B have wildly different signals for the same light phase. After determining the location, we set the motorized stage to different speeds to vary the correlation time and start continuous wavefront shaping. It is observed that the shorter the correlation time is, the less effective continuous wavefront shaping will be, as shown in **Fig 6.7 (B)**. When the correlation time is very long, the signal can be maintained at a high level similar to the original value since it acts like a static sample. With the decreasing correlation time, the intensity starts to drop significantly as the sample moves. When the correlation time is only 14 ms, the intensity at the time of stopping moving is slightly greater than that without wavefront shaping, which indicates that wavefront shaping is still effective but not well. Once the sample stops moving, continuous wavefront shaping enhances the signal until it returns to a high level. However, the final signal intensity is still lower than the original value.

This could be caused by absorption strength differences of the phantom between the starting (A) and ending (B) locations.

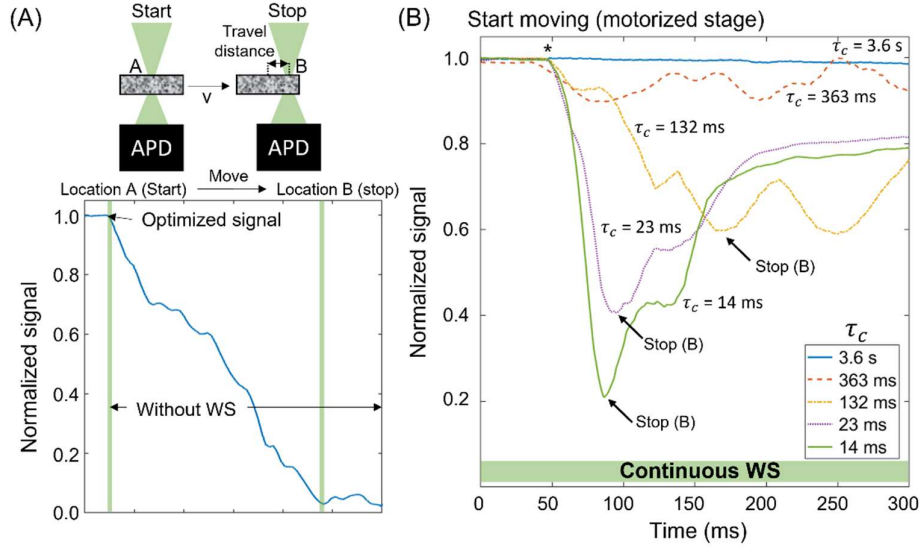


Fig. 6.7. Continuous wavefront shaping for a static sample moving on a motorized stage. The label “Stop (B)” indicates the time point at which the sample stopped moving at location B.

• Intralipid Phantoms

Besides the static sample mounted on a motorized stage, we also use the intralipid-gelatin phantoms as the dynamic sample. Due to diffusion, the speckle pattern of an intralipid-gelatin phantom constantly changes with no apparent starting and ending points. The different viscosity of the gelatin varies the speckle correlation time of a sample. We put the APD behind the sample to detect laser speckles for optimization. To obtain a reference acting like a static sample for comparison, we make a sample with gelatin at a high concentration, so it has a long correlation time (> 10 s). In this case, the reference sample can achieve enhancement similar to that for static samples such as a diffuser but note that the gelatin sample is closer to biological samples in composition. Then we test

wavefront shaping for other samples with different correlation times to compare with the reference. For temporal focusing, the time-gating window is used to isolate the signal in the targeted time gate for enhancement, as labeled in **Fig. 6.8(A)**. After optimization, sharp temporal focusing can be generated (**Fig. 6.8(B-D)**), but the temporal peak becomes broader and weaker with a shorter correlation time of the sample (**Fig. 6.8(D)**). Then the isolated signal from the time-gating window is plotted to observe the trend in **Fig. 6.9**. As shown in **Fig. 6.9(A-C)**, the signal stayed at a high level for samples similar to the static sample and those with long correlation times. However, when the correlation time is decreased to 25 ms, the average optimized intensity starts to drop significantly with considerable variation, as shown in **Fig 6.9(D)**. Wavefront shaping becomes ineffective when the correlation time is below 2 ms (**Fig 6.9(E-F)**). Therefore, in the current setup, the system can perform wavefront shaping on dynamic samples with a correlation time of around 2 ms with moderate enhancement when the SNR is not an issue.

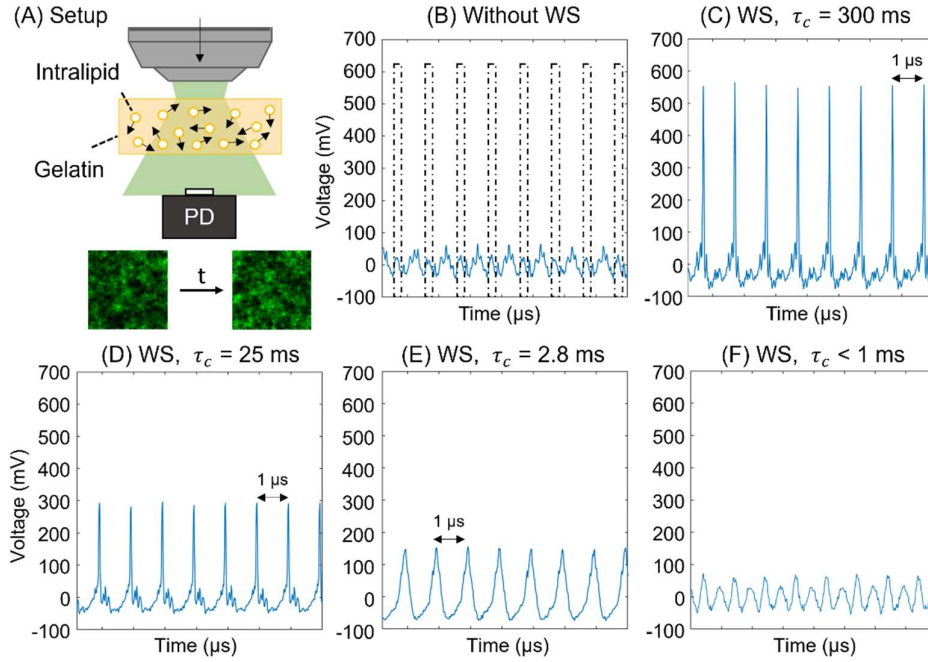


Fig. 6.8. Temporal focusing through scattering samples with different correlation times. (A) Dynamic sample made by gelatin-intralipid phantom (B) The windows marked by dash-dotted lines in the figure indicate the time gate in which the signal is used as feedback for the optimization. (B-C) Sharp peaks can be generated after wavefront shaping for samples with long correlation times. (D) When the correlation time is shorter, temporal focusing is less effective, and broader peaks with lower intensities are generated.

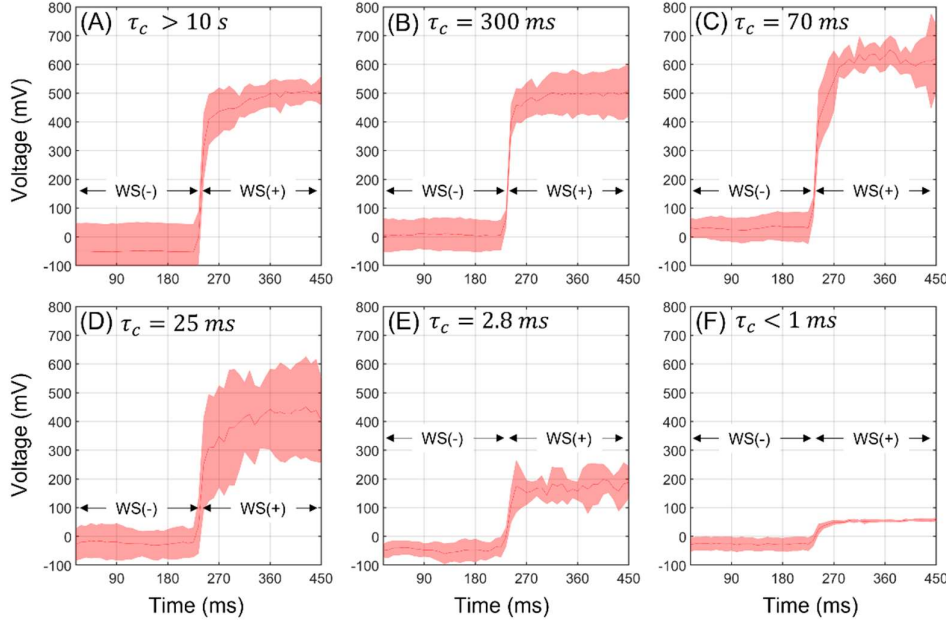


Fig. 6.9. Performance of wavefront shaping to focus light behind dynamic intralipid phantoms with different correlation times. (A-C) Like a static sample, the signal can be kept at a high level for a long correlation time. (D-E) When the correlation time decreases, the signal decreases to a low level after wavefront shaping and varies significantly. (F) Wavefront shaping becomes ineffective when the correlation time falls below 1 ms so that no significant enhancement can be observed. WS (-): Without wavefront shaping. WS (+): Continuous wavefront shaping.

6.3.3 Continuous Wavefront Shaping on Dynamic Samples for Fluorescence Enhancement

- *Fluorophore behind Intralipid Phantoms*

The advantage of using feedback-based wavefront shaping is its ability to enhance incoherent light directly. By using fluorescence as feedback, we can generate temporal focusing for fluorescence signals from Nile red particles or quantum dots (without through scattering media) with broader temporal peaks due to fluorescence lifetime (**Fig. 6.10**). This makes it possible to directly use fluorescence as the guide to enhance its intensity in disordered media. To

investigate the speed of continuous wavefront shaping on fluorescent samples, we put fluorescent particles below a scattering sample ($l_t \sim 500 \mu\text{m}$; thickness: $550 \mu\text{m}$) with a thickness of 1 transport mean free path and measure fluorescence in the transmission setup. Most excitation light becomes defocused on the sample plane. To get enough SNR for the experiment, only the excitation light is configured to suffer from multiple scattering, while fluorescence is not, as shown in Fig. 6.12. As shown in Fig. 6.11, fluorescence can be enhanced with continuous wavefront shaping and maintained at a high level when the sample acts like a static sample ($> 1.1 \text{ s}$). The signal is stable but slightly lower when the correlation time is longer than 55 ms. However, the signal becomes unstable and weak when the correlation time falls below 5 ms, and wavefront shaping becomes ineffective in cases below 2 ms. This threshold value of the correlation time for wavefront shaping using fluorescence as feedback to work is higher than that for the previous laser experiments, which is likely due to the lower SNR and fluorescence lifetime.

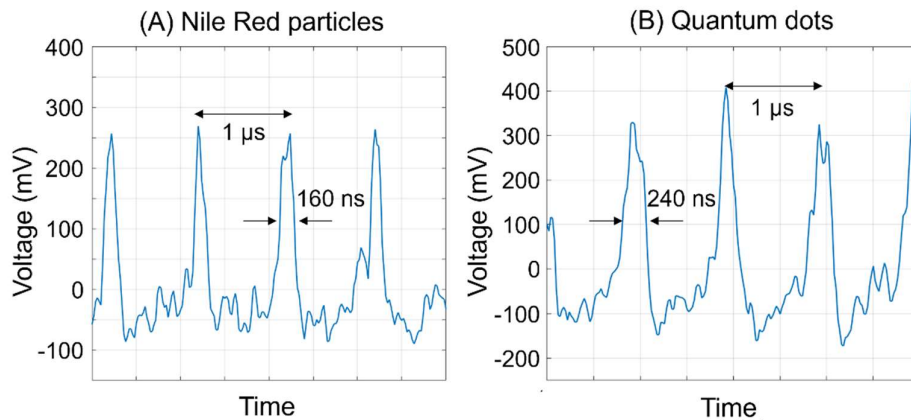


Fig. 6.10. Temporal focusing for fluorescence signal without scattering media. Without dynamic scattering media, the width of fluorescence temporal focusing is (A) 160 ns for Nile red particles and (B) 240 ns for quantum dots, which is wider than laser light (Fig. 6.1(B)).

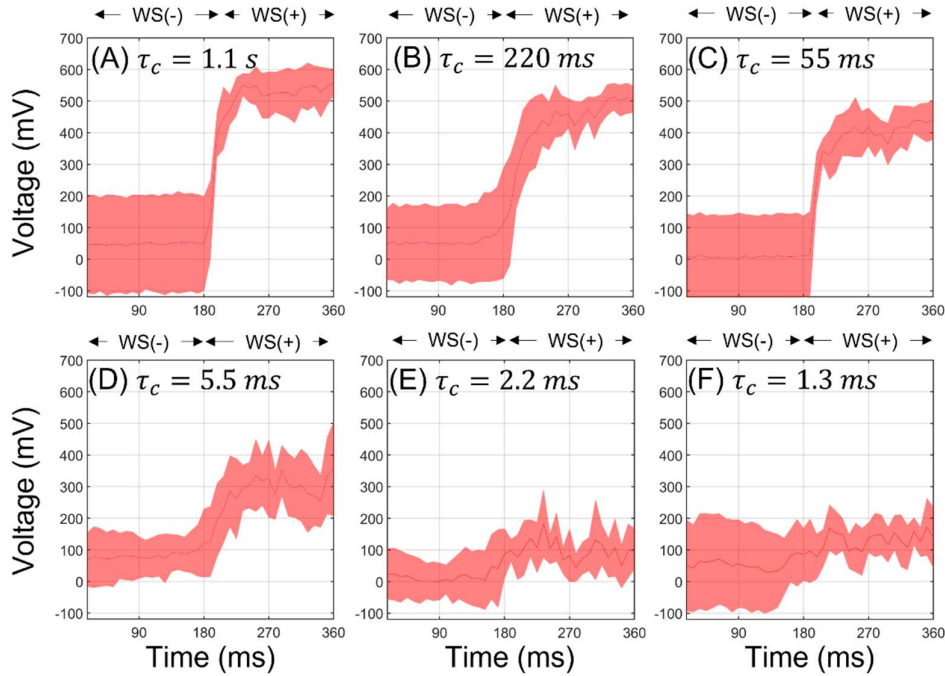


Fig. 6.11. Performance of wavefront shaping to enhance fluorescence signals measured behind intralipid phantoms on a motorized stage with different correlation times. (A-C) The fluorescence signal can be maintained at a high level for samples with long correlation times. (D) The signal becomes unstable and weak when the correlation time comes down to 5 ms. (E-F) Wavefront shaping is not effective when the correlation time is below 2 ms. WS (-): Without wavefront shaping. WS (+): Continuous wavefront shaping.

- *2D Imaging behind the Intralipid Phantom*

For 2D fluorescence imaging with point scanning, the memory effect allows 2D scanning with the same optimized phase [64] in a small region. However, since a dynamic sample keeps changing the scattering field, the memory effect is no longer effective with time. Continuous wavefront shaping is required to recover the signal at a different location. Here we scanned the sample by a 2D motorized stage and formed 2D images (**Fig. 6.12 (A)**) to investigate the performance of continuous wavefront shaping in 2D fluorescence imaging of dynamic samples. Each pixel takes around 150 ms to image, including the time for the motorized stage to stabilize and wavefront shaping while directly using fluorescence as

feedback and signal measurements. The total time for completing each image is around 2 min. As shown in **Fig. 6.12 (B-D)**, the signal can be enhanced when the sample acts like a static sample or has a long correlation time after applying continuous wavefront shaping. However, when the correlation time of the sample decreases further, the fluorescence intensity becomes weak but still higher than that without wavefront shaping measured under the same condition (**Fig. 6.12(F)**). When the sample moves at high speed with a correlation time below 2 ms, the image is not significantly different from that without wavefront shaping (**Fig. 6.12(G-H)**).

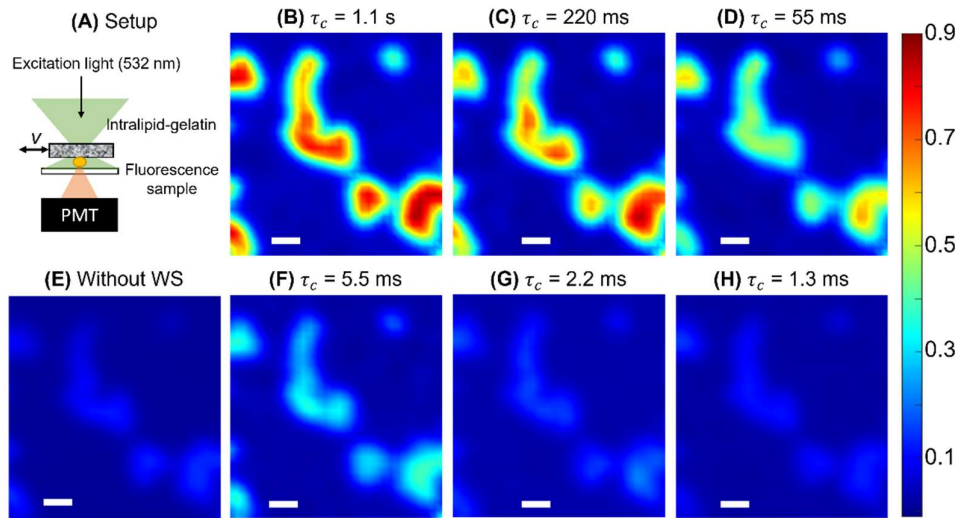


Fig. 6.12. 2D imaging of fluorescent particles behind dynamic scattering media with different correlation times after wavefront shaping. (A) The fluorescent particles are put below the intralipid-gelatin (scattering media), and fluorescence is measured in the transmission setup. (B-D) The representative signal (in the bright regions) becomes higher after wavefront shaping for samples with a long correlation time, and (F) becomes weaker and unstable but still higher than the one (E) without wavefront shaping when the correlation time is below 5 ms. (G-H) The signal becomes very weak, similar to (E), the one without wavefront shaping when the correlation time is below 2 ms. Scale bar: 15 μm .

6.3.4 *Ex Vivo* Measurements

- *Laser Speckle behind Chicken Breast Tissues*

We use chicken breast tissues to represent *ex vivo* biological samples to test the system for laser speckle enhancement in transmission measurements. We cut bulk tissues into slices of different thicknesses, as shown in **Fig. 6.13**. Each tissue slice is sandwiched between two coverslips to maintain the thickness. We use a motorized stage to change the correlation time by moving the sample at different speeds. To optimize the signal, we measure laser speckles behind samples with a camera or an APD about 6 cm away from the samples and use the signal as feedback for wavefront shaping. As shown in **Fig. 6.14**, more multiple scattering events expected in a thicker tissue yield more minor speckles. We track the optimization process over time, as shown in **Fig. 6.15**, and then retrieve optimized signals during continuous wavefront shaping for analysis. The process is repeated for chicken tissues with three different thicknesses, each with two different correlation times. With the same incident laser power (8 mW), the transmitted signal will vary for different thicknesses due to absorption and scattering. As shown in **Fig. 6.16**, for a thin tissue with a thickness of 560 μm , the optimized signal drop dramatically with a decreasing correlation time, while the optimized signal intensities in thick samples (980 μm and 1500 μm) are almost unchanged with varying correlation time. The different trends should be related to the speckle size and speckle pattern in tissue slices with varying thicknesses. Note that the change in correlation time here is induced by moving a sample on the lateral dimension. When tissue is thin, the scatterer distribution in the tissue tends to be more heterogeneous across the lateral dimension, as implied by the speckle image in **Fig 6.14**, which makes it more challenging for

continuous wavefront shaping to keep up with fast changes in scatterer distribution as the sample moves. In contrast, when tissue is thick, the scatterer distribution in the tissue appears more homogeneous across the lateral dimension, as suggested in **Fig 6.14**, which makes it easier for continuous wavefront shaping to keep up with slow changes in scatterer distribution.

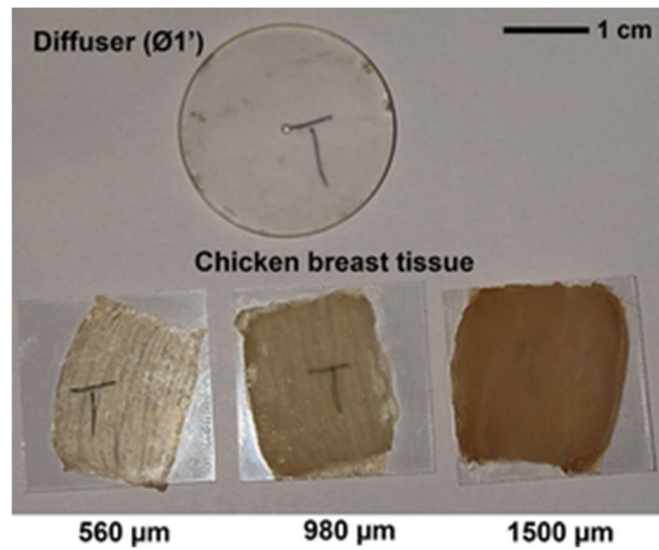


Fig. 6.13. Chicken breast tissue slices with different thicknesses and a commercial diffuser for comparison.

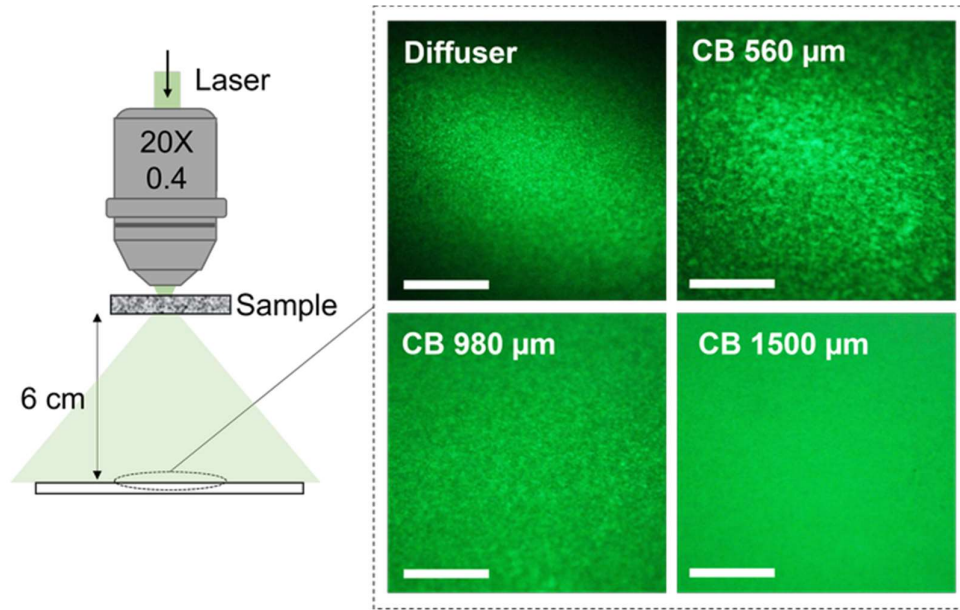


Fig. 6.14. Laser speckles are generated behind chicken tissues with different thicknesses. Speckle photos are taken 6 cm behind the samples. The speckle size becomes smaller and more homogenous as the chicken tissue gets thicker. The legends “CB” indicate chicken breast tissues with thickness values. CB: Chicken breast. Scale bar: 1 cm.

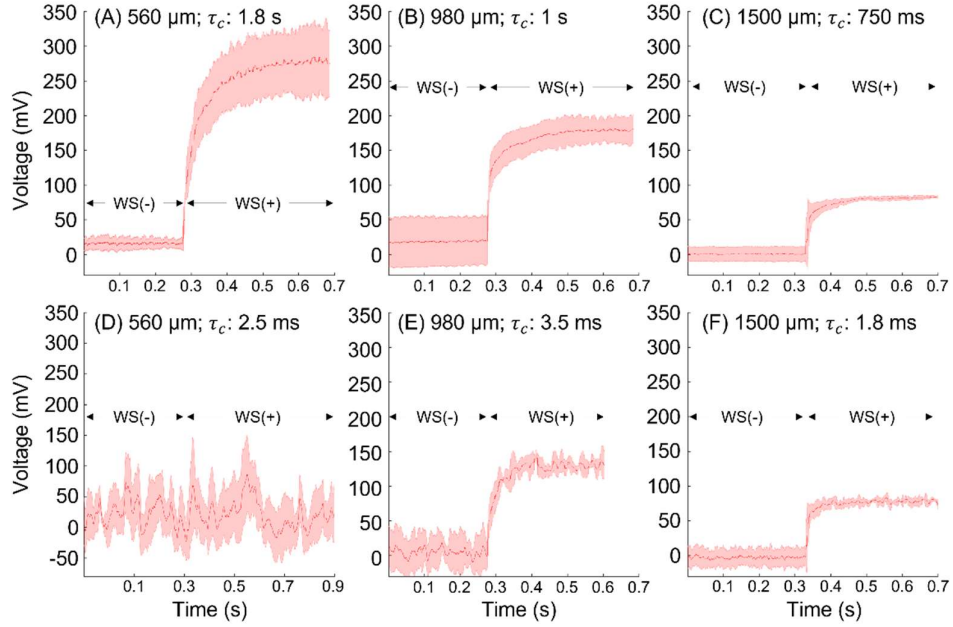


Fig. 6.15. Performance of wavefront shaping for enhancing laser light behind chicken breast tissues with different correlation times. (A-C) The signal can be enhanced for both thin and thick tissues with long correlation times. (D) Wavefront shaping becomes ineffective for a thin sample with a short correlation time. (E-F) For thick tissues with a short correlation time, wavefront shaping still works. WS (-): Without wavefront shaping. WS (+): Continuous wavefront shaping.

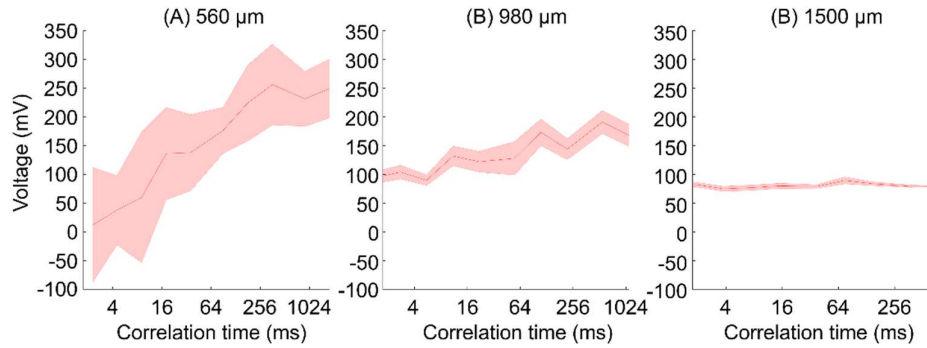


Fig. 6.16. Optimized signal intensity after wavefront shaping behind a chicken breast tissue as a function of correlation time. The optimized signal is retrieved from the optimization curve (Fig. 6.15) in the period of continuous wavefront shaping. (A) The optimized signal drops significantly for a thin sample with a shorter correlation time. (B-C) For thick tissue, the optimized signal is stable and unaffected by changes in correlation time.

- *Fluorophore behind the Chicken Breast Tissue*

To study fluorescence enhancement *ex vivo*, we put fluorescent particles behind sliced chicken breast tissues and measured fluorescence emission in the transmission setup. Different from laser speckle measurements, fluorescence signals are much weaker, which could cause the poor performance of high-speed feedback-based wavefront shaping. Inspired by the lock-in method described in **Chapter 5**, a 1-MHz bandpass filter was created by combining a 1-MHz high-pass filter (EF517; Thorlabs) and a 1-MHz low-pass filter (EF508; Thorlabs) with a bandwidth of 0.75 MHz is used to decrease the noise by isolating the signal with a narrow frequency range as shown in **Fig. 6.17**, and the time-gated enhancement is performed on the isolated signal (**Fig. 6.17(B)**). After wavefront shaping, the 1-MHz sine wave is enhanced, and the crest of the waveform is synchronized with the time gate (**Fig. 6.17(C)**). To detect the temporal focusing after wavefront shaping, we detect the signal without the 1-MHz bandpass filter in a different path. As shown in **Fig. 6.17(F)**, temporal focusing is formed by the time-gated signal as feedback after wavefront shaping with 1-MHz filtering. The performance of temporal focusing is not as good as using the whole signal as feedback as in **Fig. 6.1** since it only takes advantage of part of the signal. The benefit of time-gating enhancement with bandpass filtering is the ability to work with weak signals in the case of relatively thick tissues. For the tissue with a thickness of 500 μm (**Fig. 6.18(A)**), both methods (whole signal enhancement and 1-MHz bandpass enhancement) can enhance the fluorescence signal behind the chicken breast tissue. However, for another tissue with a thickness of 1200 μm , the approach of using the whole signal as feedback will fail due to the weaker signal submerged in noise, while the approach of using a 1-MHz signal as

feedback can still detect a decent signal and yield considerable enhancement by rejecting out-of-band noise (**Fig. 6.18(B)**). We also scan the 1D profile of fluorescent particles (**Fig. 6.19(A-B)**) through the tissue with varying correlation time obtained by moving the tissue on a motorized stage. As shown in **Fig. 6.19(C-D)**, with a decreasing correlation time of the sample, the signal will drop down to the level without wavefront shaping (< 2.3 ms for the tissue with 500- μm thickness, < 11 ms for the tissue with 1200- μm thickness). However, the signal does not significantly change with varying correlation time for the tissue with a 1700- μm thickness (**Fig. 6.19(E)**). For thin samples (with thicknesses of 500 μm & 1200 μm), wavefront shaping yields higher enhancement in the case of long correlation time since wavefront shaping is fast enough to catch up with the slowly changing speckle pattern. However, the enhancement significantly degrades when the correlation time decreases because of the rapidly changing speckle pattern. On the other hand, for the thick sample (1700 μm), the enhancement seems not to seem affected much by sample motion due to the high homogeneity of the speckle distribution in a large region of the illumination area. The result is similar to the laser speckle enhancement behind thick chicken breast tissues (**Fig. 6.16**).

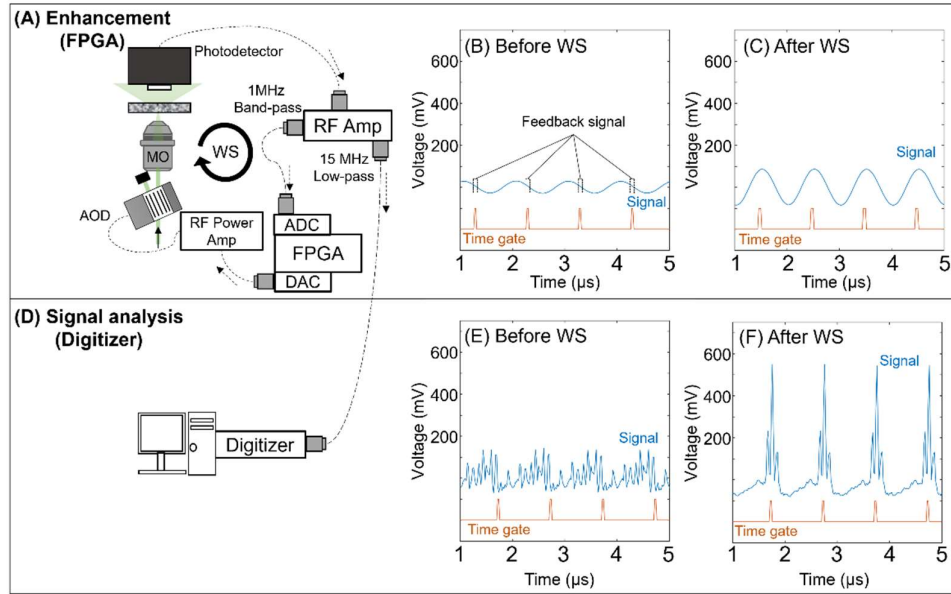


Fig. 6.17. Feedback-based wavefront shaping for temporal focusing with 1-MHz filtering. (A) The 1-MHz isolated signal is used as the feedback for temporal focusing. (B) After wavefront shaping, the 1-MHz signal will be enhanced, and the crest of its waveform will be synchronized with the time gate. (D) The signal without 1-MHz filtering is used to form temporal focusing. (E-F) After wavefront shaping with 1-MHz filtering, the temporal focusing can be formed within the time gate.

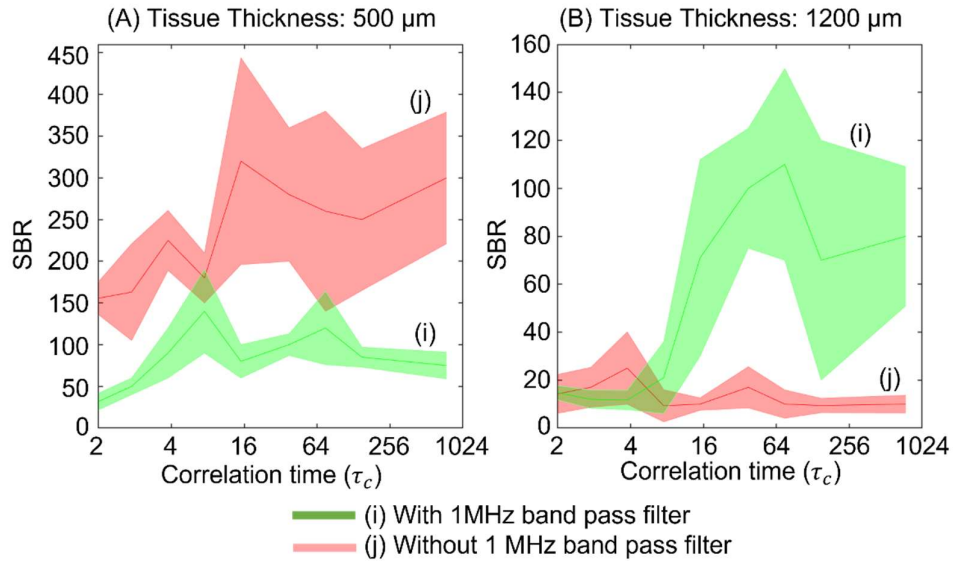


Fig. 6.18. Comparison of two methods using the whole signal or filtered 1-MHz signal as feedback for wavefront shaping. (A) Both methods can enhance the signal for a thin tissue with 500- μ m thickness. The whole signal enhancement has a higher signal since the performance of temporal focusing is better. (B) For a thick tissue with 1200- μ m thickness, only the filtered 1-MHz signal for wavefront shaping is effective. The y label “SBR” represents the signal-to-background ratio.

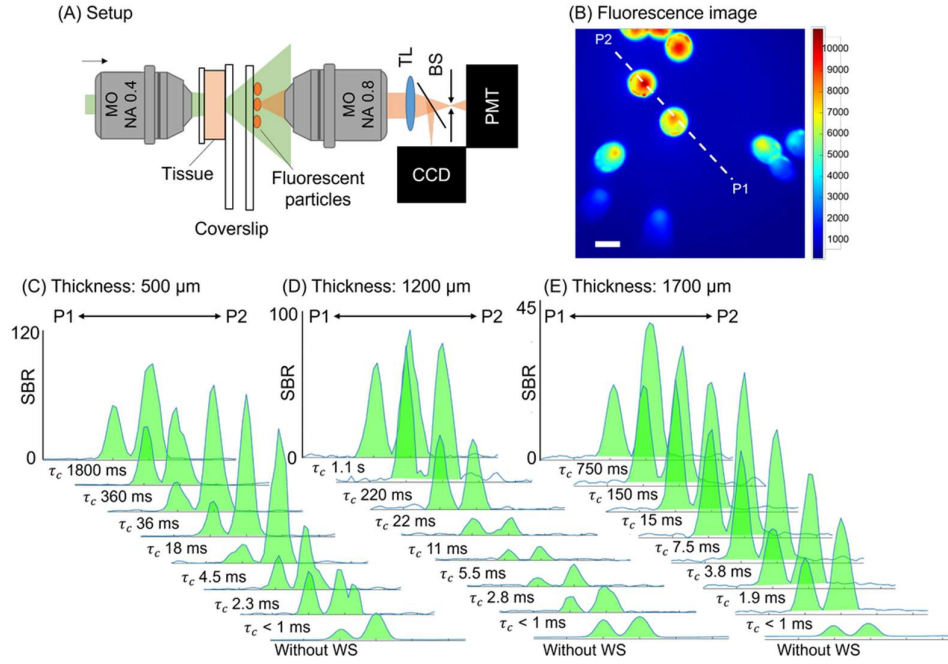


Fig. 6.19. One-dimensional profile of fluorescent particles behind chicken breast tissues with different correlation times after wavefront shaping. (A) In the setup, fluorescent particles are placed on the coverslip and put behind the chicken breast tissue, sandwiched by two coverslips. (B) A commercial camera took the fluorescence image, and the white dash-line shows the region along which 1D profiles are obtained by scanning. Scale bar: 10 μm . (C-E) The 1D profile across the line from P1 to P2, as shown in (B), after continuous wavefront shaping when the tissue in the middle is moved on a translational stage to create a series of different correlation times. SBR: Signal-to-background ratio; TL: Tube lens; BS: Beam splitter.

6.4 Discussion

- *Time-Gating Signal*

For temporal focusing, the signal within a narrow time window is required to be isolated for analysis. In this experiment, we use digital time-gating isolation to retrieve the signal at a specific time. Since temporal focusing is time-specific, one can use a pulse laser or AOM-modulated light that only allows the light to go through the AOD within a fixed time window. In this way, a single-pixel photodetector with a high bandwidth is only required for optimization but not required to measure the enhanced signal; then, both commercial spectrometers and standard scientific CCD/CMOS cameras can be used to measure optical spectra or perform 2D imaging. This can avoid the scanning process when taking spectra or 2D images with a 1D or 2D camera, which can be used in combination with the cameras' triggering function and exposure time set to improve the signal-to-noise ratio further.

Since the time-gating signal is used as the feedback to get temporal focusing, a high-power pulsed laser could benefit the application of *in vivo* measurements as long as the laser energy is limited below the safety threshold value. The high-power light source with repetitive high-energy pulses lasting only for a short time can increase the SNR for fluorescence enhancement.

- *Temporal Focusing and Magnitude Enhancement*

With the combination of a bandpass filter and a lock-in amplifier (**Chapter 5**), the influence of noise can be significantly reduced, which makes feedback-based wavefront shaping work for highly scattering samples at the cost of relatively low enhancement. For temporal focusing used in this study, noise strongly affects the wavefront optimization process, and wavefront shaping is easy to fail in the case

of low SNR. Therefore, compared to spatial focusing, temporal focusing achieved with wavefront shaping can provide higher enhancement. The choice of feedback is based on the trade-off between SNR and speed. The high-speed method based on temporal focusing for laser light can achieve higher enhancement in a short time window. For fluorescence or Raman scattering enhancement, the wavefront shaping method based on lock-in amplification can be used to detect weak signals at the cost of lower enhancement, as demonstrated in **Chapter 5**.

- *Degrees of Freedom*

The degrees of freedom of AOD are much lesser than other 2D modulators, such as SLM or DMD, which limits the maximum enhancement factor. Unlike SLM and DMD, which can achieve a maximum enhancement greater than 10^6 [68], there is no significant difference in enhancement factor even when the DOF of the AOD goes above hundreds of degrees of freedom [75]. While the enhancement factor increases with the number of degrees of freedom, each iteration in feedback-based wavefront shaping will take longer with more degrees of freedom. Therefore, it is necessary to speed up iterative optimization by sacrificing the enhancement factor using AOD for wavefront shaping. This trade-off between speed and enhancement factor can be made according to the scattering correlation time of the sample.

- *FPGA-Based Wavefront Shaping*

FPGA provides highly parallel computing for waveform generation and signals analysis that significantly speeds up the optimization. The integrated modules,

such as DAC and ADC, can replace the function generator and digitizer to control the AOD and receive the signal. Therefore, with the closed-loop setup, the whole optimization process can function rapidly without external intervention. Besides, the output signal generated by FPGA in nanoseconds can be easily used to control other devices with precise timing for custom requirements. However, it should be noted that the limited resources of the FPGA could make it necessary to sacrifice performance for speed or vice versa.

- *Application*

The high-speed feedback-based wavefront shaping technique developed in this study could be potentially used for *in vivo* tissue measurements, in which the speckle correlation time is short due to blood flow and breathing. When used for fluorescence measurements, its disadvantage in SNR could be compensated by using a pulse laser or AOM-modulated laser and then isolating the time-gating signal after wavefront shaping. Moreover, the setup involving AOD and FPGA could be integrated into a portable device for mobile use.

6.5 Conclusion

This study demonstrates high-speed feedback-based wavefront shaping with FPGA acceleration for spatiotemporal focusing. The system can focus the light behind scattering media on a millisecond scale for laser light and fluorescence enhancement.

Chapter 7

Fast Depth-Resolved Imaging Based on Radiofrequency Tagging

7.1 Introduction

Fluorescence microscopy has become an attractive tool in diagnosing human epithelial precancers due to its capability to detect fluorophores associated with physiological and structural changes in epithelial tissues non-invasively [8, 136-138]. Moreover, the depth distribution of these biological fluorophores is related to the progression of diseases [8, 139-141]; thus, depth-sensitive fluorescence measurements are essential to enhance these layered tissues' diagnostic outcomes. In such measurements, fast imaging is vital to suppress the motion artifacts and acquire blur-free images for visualizing live tissues *in situ* [142, 143]. Therefore, high-speed depth-sensitive fluorescence measurements are desired to improve the diagnostic performance of epithelial precancers.

Several reports have been speeding up depth-sensitive measurements in the literature, as surveyed below. Badon et al. proposed multi-Z confocal microscopy using multiple reflection pinholes axially distributed in the detection space to conduct simultaneous multiplane imaging without the need for axial scanning to improve the imaging speed, in which each set of pinhole and photodetector records the signal at a different depth in a sample [144]. The limitation of this approach is that more pinholes and photodetectors will be needed when

modifying the system to measure signals from more depths. The upper limit of the system in measurement throughput is restricted by the number of pinholes and corresponding photodetectors, and more sets of pinholes and photodetectors would increase the system's complexity. Duocastella et al. presented another method to conduct depth-resolve imaging at a high acquisition rate by combining an acoustic-optical scanner and an acoustic varifocal lens. The acoustic-optical scanner is used to generate light-sheet illumination with high-speed plane-to-plane switching to obviate mechanical movement in the depth direction. The acoustic varifocal lens in the detection path extends the depth-of-field, of which the signal-to-background in focus will be decreased as the axial scanning range increases [145].

In the last decade, an approach called the frequency-division-multiplexing (FDM) technique has gained interest in fluorescence microscopy to address the speed limit since it can generate multiple optical beams in the illumination path simultaneously based on radio frequency coding using acousto-optic devices [75, 130, 146-148]. In the detection path, a single-pixel photodetector is applied for signal integration, and the detected signal can be recovered by digitally processing the detected frequency-encoding signals. However, all these FDM techniques so far are still limited to imaging in the lateral dimension to our best knowledge.

In this study, to utilize the FDM method in axial imaging for depth-sensitive fluorescence measurements, we propose a novel microscope configuration to generate multiple frequency-encoding focal spots arranged in the axial dimension by combining remote focusing and AOD-shifted frequency generation. Specifically, multiple beams generated by digitally synthesized beat frequency

multiplexing are focused on different depths simultaneously to obtain depth-related information. Meanwhile, imaging information at different depths can be detected by a single-pixel photodetector simultaneously, which eliminates the need to detect signals from different depths sequentially to accelerate depth-resolved imaging. In addition, it would be convenient to improve the system by using more frequency-encoding beams without increasing the number of photodetectors or system complexity.

7.2 Experimental Principle and Apparatus

7.2.1 Principle of AOD-Depth Scan

The purpose of generating multiple focal spots along depth using AOD is to retrieve signals from different depths simultaneously to speed up depth-sensitive measurement. It is well known that an AOD can generate multiple beams leading to several focal spots in the lateral dimension. We need to convert the lateral distribution of laser beams to multiple focal spots along the axial dimension for depth-sensitive measurements. The details of the lateral-to-axial conversion are described below.

- ***Generation of Multiple Focusing Spots along the Axial Dimension with Remote Focusing***

AOD can generate multiple frequency-shifted beams with different angles by light diffraction. The diffraction angle is related to the driving radio frequency and light wavelength under the Bragg condition. To perform quick depth-sensitive measurements, these beams distributed laterally need to form focal spots along the axial dimension [149]. In a similar effort to achieve axial scanning,

a reference mirror can be moved along the axial dimension to create a series of focal spots at different depths in a sample sequentially in the literature, as shown in **Fig. 7.1**. Alternatively, multiple focal spots on the same focal plane as shown in **Fig. 7.2(A)** can be translated to the periphery of the objective's back aperture as shown in **Fig. 7.2(B)** to create an angle between the light propagation direction and the objective's optical axis. Then a mirror on the focal plane is tilted to reflect beams, as shown in **Fig. 7.2(C)**, which creates a gradient of axial positions of focal spots. These focal spots are imaged onto a sample at a different depth as desired, as shown in **Fig. 7.2(D)** [150].

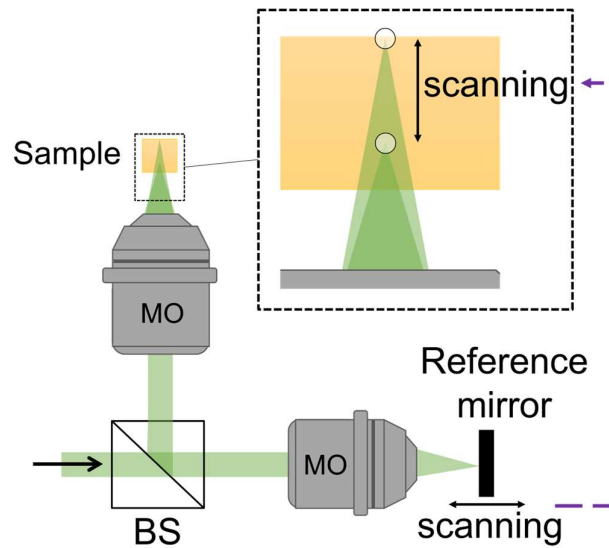


Fig. 7.1. Remote focusing with a steering mirror. Due to the conjugation of the reference plane and the sample plane, changing the position of the reference mirror will change the depth of the focal spot in the sample. MO: Microscope objectives; BS: Beam splitter.

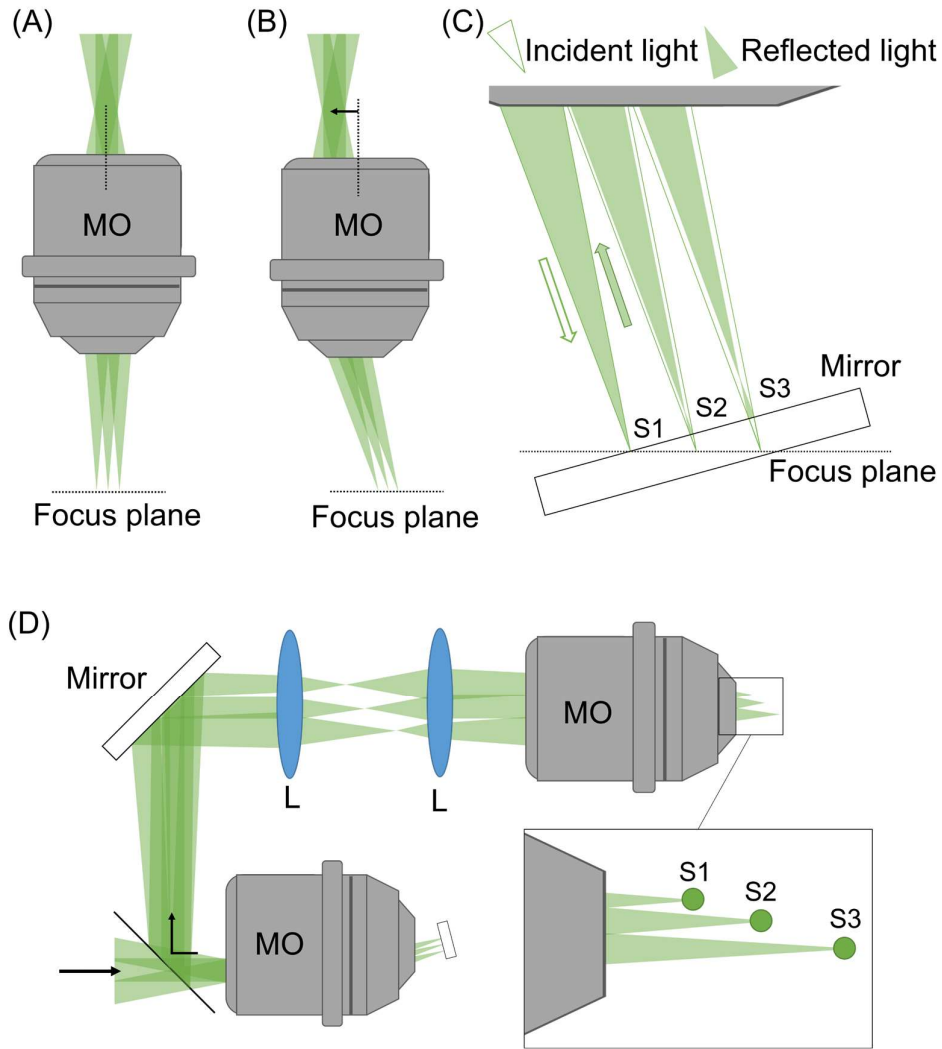


Fig. 7.2. Modulation of focal depth with a tilt mirror for generation of focus spots at different depths. When light is translated from (A) the center to (B) the periphery of the back aperture of the objective, the focusing beams after the objective form an angle relative to the optical axis of the objective. (C) With the angle caused by the beam translation, a tilted mirror can reflect the light to create a gradient of axial positions of focal spots. (D) These focal spots are imaged onto a sample, each at a different depth.

- ***Beat Frequency Generation from the Interference between a Focal Line and Multiple Focal Spots***

Each focal spot at a different depth has a different frequency shift stemming from AOD diffraction, which can be used to generate a unique beat frequency at every depth upon interference with the original laser light. To get information from different depths, a photodetector with high bandwidth can detect the frequency multiplexing signal from the sample, on which the beat signal can be reconstructed by performing FFT. The magnitude of each beat frequency will represent the measurement at a different depth.

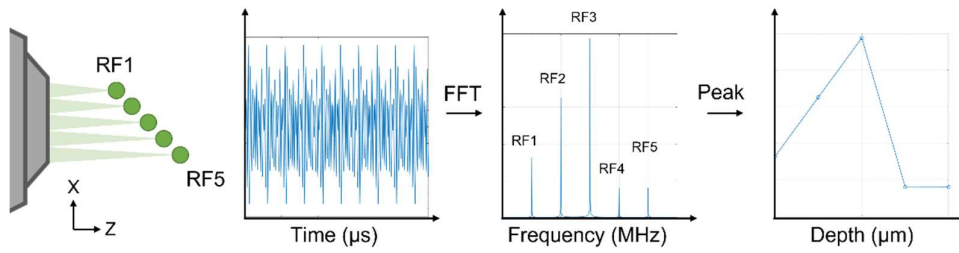


Fig. 7.3. Generation of depth-dependent beat signals. With multiple focal spots by AOD and remote focusing, the interference generated at each depth axis yields a signal with a specific beat frequency. RF: radio frequency.

7.2.2 Experiment Apparatus

The optical setup for generating multiple focal spots at different depths, each with a unique beat frequency, is shown in **Fig. 7.4**. A 532-nm diode-pumped solid-state laser (3900S laser, Newport Spectra-Physics Ltd, USA) beam is split into two by a polarizing beam splitter, and a half-wave plate controls the splitting ratio. One beam is directed to an acousto-optic frequency shifter (AOFS) while the other maintains the light frequency without any frequency shift. Both beams are directed into an acousto-optic deflector (AOD) (AODF 4090-7, Gooch &

Housego PLC, UK), which is driven by a multi-tone RF signal generated by an arbitrary waveform generator (AFG3101, Tektronix Inc., USA), and then diffracted to different angles corresponding to the driving radio frequencies (RF). To generate the beat signal, two arms (AODF diffracted laser beam and non-AODF laser beam) are superposed to form interference. However, since the frequency shift is only caused by AODF, every AOD-diffracted light beam from one arm would yield the same 40-MHz beat frequency if interfering with the corresponding beam from the other arm. Therefore, to generate different beat frequencies, one of the arms is laterally reversed, as shown in **Fig. 7.5**. In this way, the interference between two arms for each diffracted light beam after AOD will produce a different beat frequency. The beat signals match the driving frequency to AOD, as shown in **Fig. 7.6**.

Once multiple beams, each with a different beat frequency, are generated, the distribution of the focal spots created by focusing these beams is transformed from the lateral direction to the axial direction by use of a tilted mirror in the same manner as in **Fig. 7.2**. These beams with different beat frequencies are focused into a sample at different depths.

In the detection path, fluorescence signals from the different depths within the sample are collected by another objective and focused onto a photomultiplier tube (PMT, 77348; Newport). The microscope objective is placed in such a way that the surface of the objective's front aperture is parallel to the line formed by all laser focal spots. This way, since all fluorescence can be collected from the same distance away from the objectives, we can use a commercial slit to filter fluorescence light without tilting to improve spatial resolution. A PMT will then record the filtered light, and the analog output from

the PMT is filtered by a 1-MHz high-pass filter, amplified by a preamplifier (DC-350MHz; Stanford Research Systems, USA), and digitized by a digitizer. FFT then processes the measured signal in MATLAB to retrieve the magnitude of each frequency component, one corresponding to a unique depth.

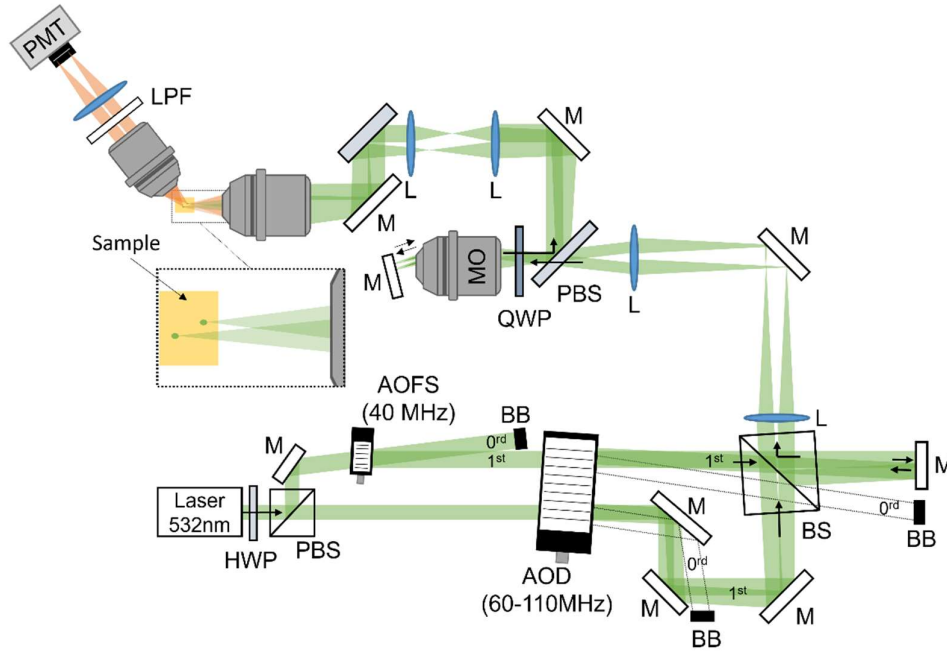


Fig. 7.4. Schematic of the optical setup for generating multiple focal spots with different depths by remote focusing. L: Doublet lens; BS: Beam splitter; PBS: Polarization beam splitter; AOD: Acousto-optic deflector; AOFS: Acousto-Optic frequency shifter; HWP: Half-wave plate; QWP: Quarter-wave plate; DM: Dichroic mirror; PMT: Photomultiplier tube; MO: Microscope objective; M: mirror; BB: Beam block.

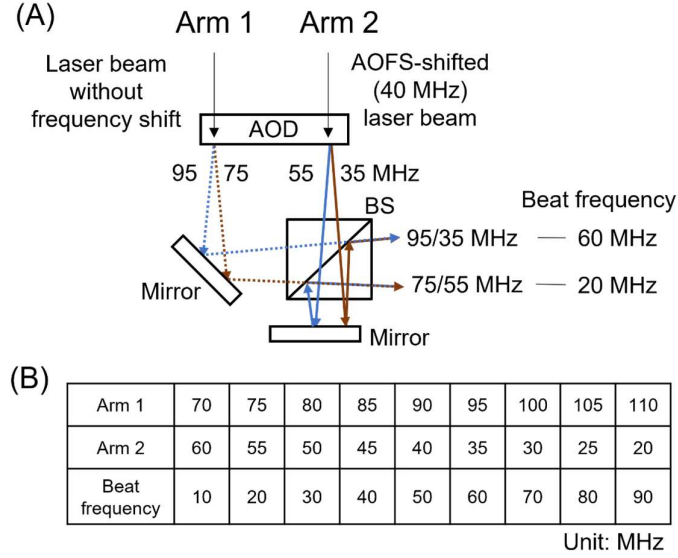


Fig. 7.5. Beat signal generation by the interference between two arms. One arm includes laterally reversed AOFS-shifted beams. For one arm, the diffracted light contains a frequency shift range from +60 to +110 MHz right after AOD. AOFS induces an extra -40 MHz frequency shift for the other arm to the output laser beams. The superposition of two arms will generate beat signals. If the two arms are not modified, only one 40-MHz beat signal will be generated. (B) Multiple beams with different beat frequencies can be generated by laterally reversing one of the arms.

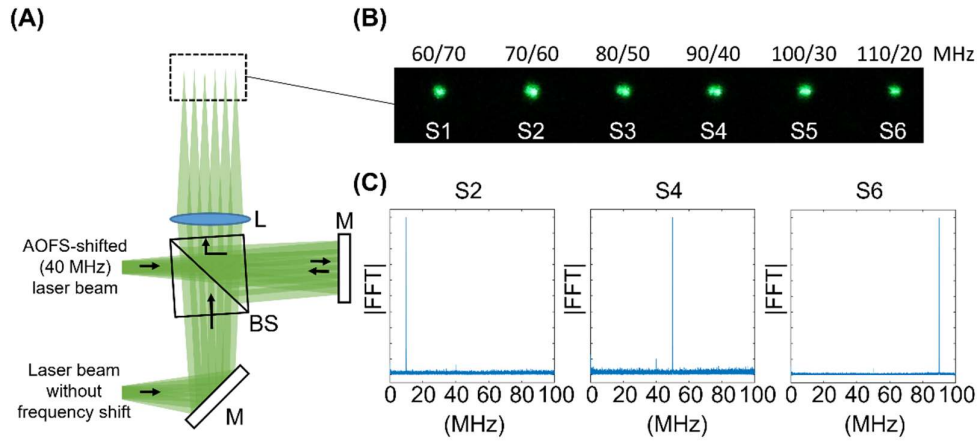


Fig. 7.6. Measurement of the beat signal by APD. (A-B) In this demonstration, a signal that contains a series of driving frequencies, including 60, 70, 80, 90, 100, and 110 MHz, is used to drive the AOD. With an extra AOFS, the second series of frequency shifts can be generated (20, 30, 40, 50, 60, and 70 MHz). The superposition of two arms generates the beat signals while one of the arms is laterally reversed. (C) The peak locations of the measured signal in the frequency domain match the driving frequencies of the AOD.

7.3 Experimental Results

7.3.1 Generation of Multiple Laser Focal Spots

A transmission setup is used to observe multiple focal spots, as shown in **Fig. 7.7(A)**. A CMOS camera records the image, and the objective in front of the camera is mounted on a 1D motorized translation stage for z-scanning. The X-Z image of multiple laser spots is shown in **Fig. 7.7 (B)**. S1 to S5 indicate the laser focal spots with beat frequencies from 75 MHz to 95 MHz with a spacing of 5 MHz. These five spots span 120 and 105 μm along the X and Z axes, respectively. By using the entire available frequency range from 10 to 90 MHz, the scanning range can go up to 240 μm and 210 μm along the X and Z axes, respectively. To investigate the spatial resolution, 1D profiles along the X and Z axes are shown in **Fig. 7.7 (C)**. The lateral and axial resolutions in terms of full width at half maximum (FWHM) are around 1.8 μm and 28 μm , respectively.

Next, we investigate the system performance by moving fluorescent particles with a diameter of 2 μm , close to the spatial resolution along the X-axis, through each focal spot and record the magnitudes of all beat frequencies retrieved by taking FFT of the signal measured by the PMT. From the Z scanning profile (**Fig. 7.8**), we can observe that the spatial resolution in the Z axis varies from 20 to 32 μm , which is close to the spatial resolution determined in laser measurements as shown in **Fig. 7.7 (C)**.

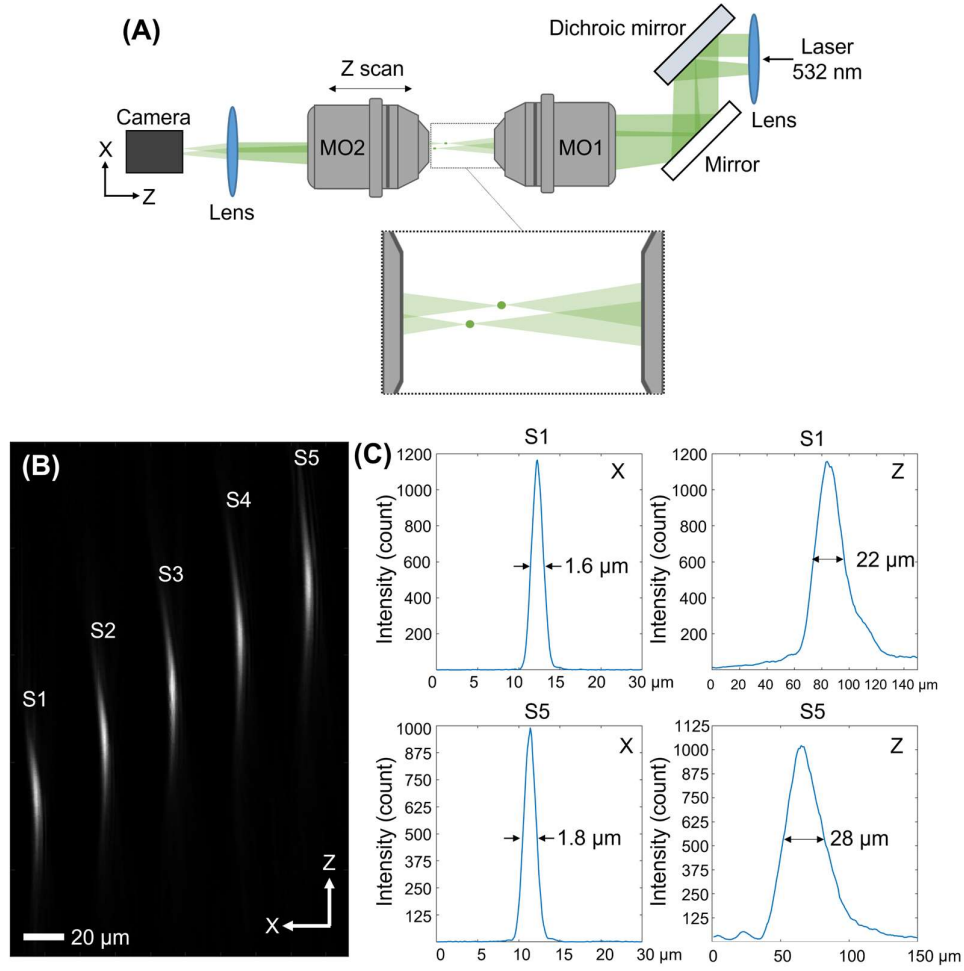


Fig. 7.7. Profile of multiple laser focal spots. Driving frequencies (75, 80, 85, 90, 95 MHz) are used to generate five laser focus spots for demonstration. (A) The transmission setup for generating (B) 2D images of the laser focal spots by scanning the second objective (MO2) along the Z axis. (C) 1D profiles of the focal spot along the X and Z axes.

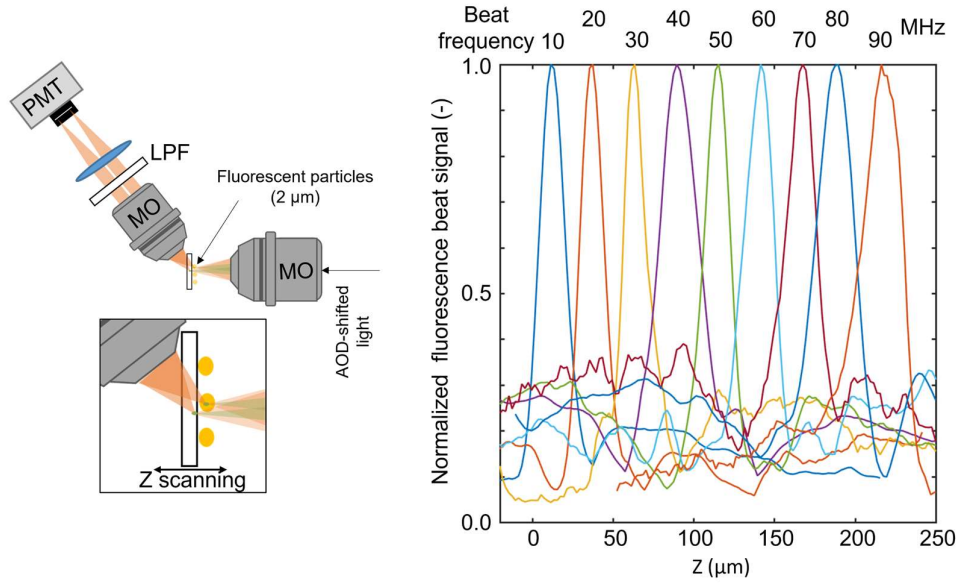


Fig. 7.8. Fluorescent profile at different depths from the focusing laser light with the different beat frequencies. The axial resolution is ranged from 20 to 32 μm .

7.3.2 Simultaneous Measurements of Depth-Resolved Signals

We can simultaneously measure fluorescence signals from different depths using multiple focal spots in the axial dimension. A fluorescent particle is used as the sample, as shown in **Fig. 7.9(A)**, and the reference image of the particle is shown in **Fig. 7.9(B)**. The multiplexing signal from the PMT can be used to reconstruct the particle image after FFT. As shown in **Fig. 7.9 (C)**, a 2D image can be generated by reconstructing the 1D image along the Z axis using FFT and scanning the sample along the Y axis when the beat frequency spacing is 10 MHz, which corresponds to around 25 μm in the Z axis. Since the particle is around 11 μm , the reconstruction image is inaccurate along the z-axis. By using a smaller beat frequency spacing, the information along Z is increasingly filled, and better images of the particle are obtained, as shown in **Fig. 7.9 (D-E)**, which is similar

to the reference image taken by a camera (**Fig. 7.9(B)**). With the same setup, we can image a larger area with multiple particles, as shown in **Fig.7.10**. The integration time is 25 μs for each line containing 10 points, corresponding to 40 kHz in measurement speed. In theory, the integration time can go down to 1 μs since the smallest frequency spacing here is higher than 1 MHz, which in practice is limited by the signal-to-noise ratio of measurements. In comparison, a commercial piezo axial scanner or electrically tunable lens (ETL) can only work with a speed of around a few hundred hertz or less. For measurements in a depth range around a few hundred micrometers, it would require more than tens of milliseconds to acquire the signal along the whole depth range. In contrast, our approach has the potential to get the multiplexing signal for the entire depth range within a very short time, possibly less than 10 μs (same as 50 kHz resonant scanning) for 20 resolvable points, when there is a sufficient signal-to-noise ratio. Potentially, 200 resolvable points with a frequency spacing of 400 kHz can be measured below 5 μs (same as 100 kHz resonant scanning) [130].

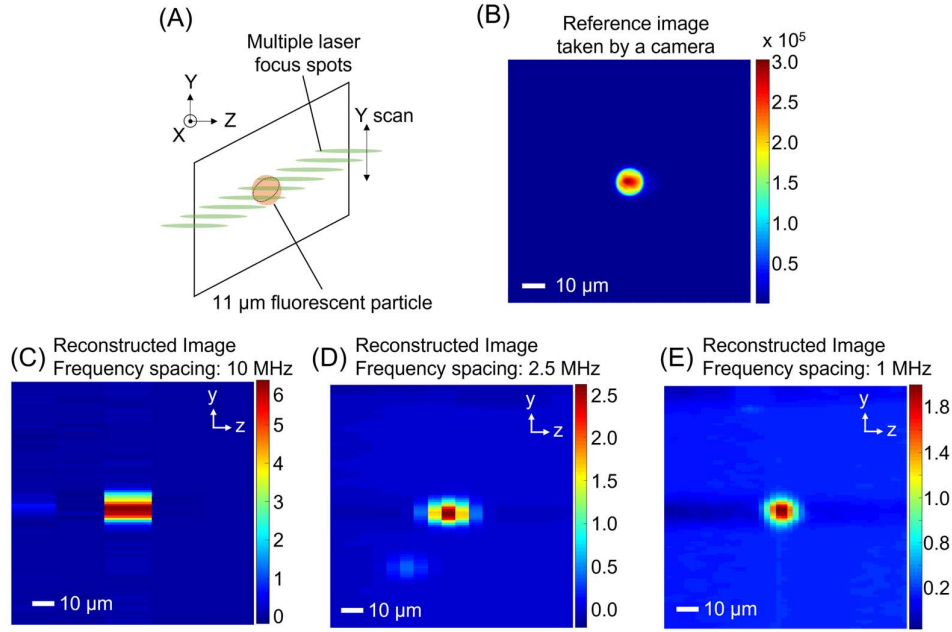


Fig. 7.9. Reconstructing fluorescent particle images in the Y-Z plane. (A) With multiple focal spots along the Z-axis, Y scanning can generate the 2D image of (B), a single fluorescent particle with a diameter of 11 μm. (C) When the beat frequency spacing is 10 MHz, the distance between two adjacent focal spots is around 25 μm. Since this spacing distance is bigger than the particle size (11 μm), the shape of the fluorescent particle is not accurate along the Z axis. (D-E) When the beat frequency spacing decreases to 2.5MHz (with a spacing distance of 6.4 μm) and 1 MHz (with a spacing distance of 2.6 μm), the reconstructed image becomes increasingly similar to the reference image taken by a camera.

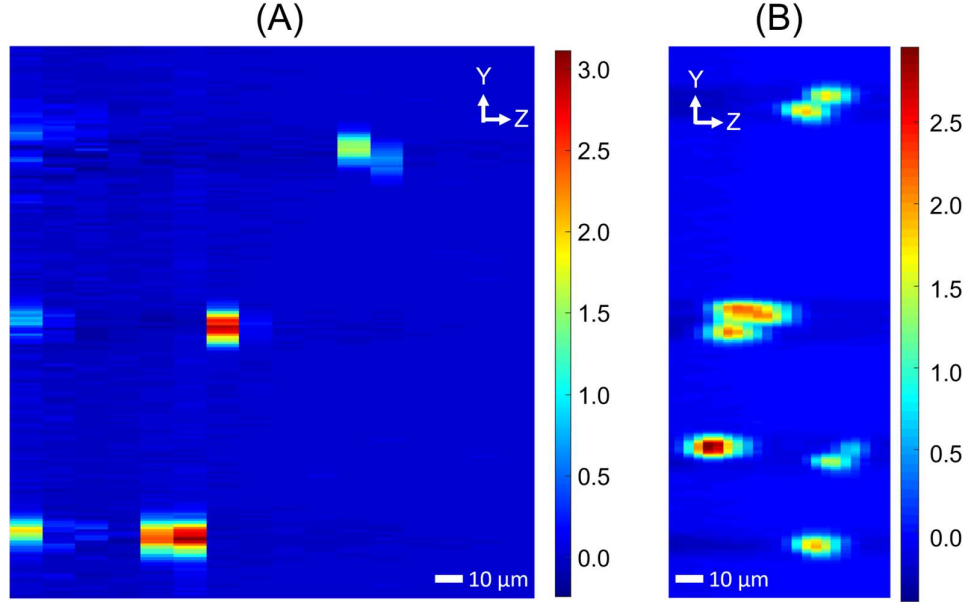


Fig. 7.10. 2D image of multiple fluorescent particles. (A) Beat frequency spacing = 5 MHz (with a spacing distance of 12.8 μm in the z-axis) (B) Beat frequency spacing = 1 MHz (with a spacing distance of 2.6 μm in the z-axis).

7.4 Discussion

7.3.3 Resolution and the Depth Range

The resolution and range of depth are correlated and affected by the AOD scanning angle and the remote focusing of the reference mirror. For resonant scanners with an extensive scanning range from 10 to 20 degrees, it can achieve high-resolution axial imaging with a fine scanning depth range [150]. However, AOD only has a scanning range of 2.5 degrees at most. Hence, due to the limited scanning angle of the AOD, the entire depth range is fundamentally small. With a fixed optical system with a specific resolution, the number of resolvable focal spots that can be well separated from each other is fixed. Therefore, to increase the measurable depth range, demagnification of the sample plane can be induced to extend the depth of field at the cost of degraded spatial resolution and cause

aberration. To enhance the performance, e.g., to increase the depth range with an acceptable resolution, an AOD with higher bandwidth (> 100 MHz) can be used to increase the scanning angle.

7.3.4 Potential Improvement

- ***Reflection setup***

In the demonstration, we show a transmission setup for fluorescence measurements. For biological samples, reflection measurements are usually preferred. Specific objectives can be selected to achieve the goal, as shown in **Fig. 7.10**, in which the working distance, case size, and microscope objectives need to be carefully considered. A long working distance and a small objective barrel are required for the illuminating objective to fit into the limited space. The NA of the objective for detection needs to be large enough to collect fluorescence, which is crucial to prevent photobleaching by short data acquisition.

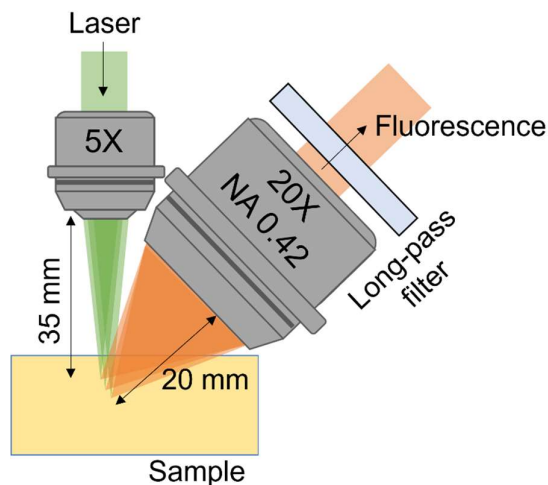


Fig. 7.11. Reflection setup for simultaneous depth-sensitive measurements. The setup is designed for a sample that requires measurements on the same side as illumination.

7.5 Conclusion

In summary, with remote reference focusing and multiple focal spots at different depths, each with a unique radiofrequency beat signal generated using a tilted mirror and an AOD, respectively, signals from different depths in a sample can be simultaneously measured with a fast single-channel detector at high speed. This method can complement the current methods for sequential depth-dependent measurements involving a piezo actuator, an electrically tunable lens, and a TAG lens, increasing effective exposure time and improving the overall measurement throughput.

Chapter 8

Conclusions and Future Directions

8.1 Conclusions

The dissertation develops techniques for wavefront shaping and simultaneous measurements of multiple depths to improve depth-sensitive spectroscopy using different strategies.

8.1.1 Feedback-Based Wavefront Shaping with Different Strategies for Improvement

For signal enhancement and increased spatial resolution in imaging through a scattering medium, we use feedback-based wavefront shaping techniques to improve performance in two aspects: (a) the signal-to-noise ratio (SNR) of the feedback signal and (b) the speed of the iterative optimization, in which essential findings are highlighted below.

(a) Increase Signal-to-Background Ratio (SBR) and Signal-to-Noise Ratio (SNR) for Improving the Performance of Wavefront Shaping

In the first study, we use spatial and spectral filtering techniques to increase the SNB of the feedback signal. The two filtering techniques work by effectively increasing the relative contribution from the target layer to the feedback signal during wavefront optimization through spatially and spectrally rejecting off-

target fluorescence light, which is substantially similar to the role of time or coherence gating. In the second study, we use the lock-in detection method to increase the SNR by generating a target signal with a specific frequency and isolating it from the background in the frequency domain. The lock-in detection method is suitable for weak signals such as fluorescence and Raman scattering in a reflection setup. The technique could be potentially used for *in vivo* measurements that often cannot be conducted in a transmission setup.

(b) Increase the Speed of the Iterative Optimization for Dynamic Samples

In the third study, we combine an acousto-optic deflector (AOD) and a field-programmable gate array (FPGA) to speed up feedback-based wavefront shaping. The decrease in time for each iteration could potentially keep up with a sample with a short correlation time. We demonstrate that high-speed feedback-based wavefront shaping with FPGA acceleration can achieve spatiotemporal focusing, which offers higher enhancement than spatial focusing alone. The system can focus light through scattering media in milliseconds for laser light and fluorescence enhancement.

8.1.2 Simultaneous Measurement of the Signal from Different Depth

In the final study, depth-sensitive spectroscopy is sped up by encoding information from each depth with a unique radio frequency. We use AOD to create multiple focal spots simultaneously, each with a different radio frequency along the axial dimension and measure the signal with a single-pixel detector. Because the depth information is encoded in the RF signal, we can reconstruct

depth-relevant information from the data measured by the single-pixel detector. This technique dramatically increases the speed of depth-sensitive spectroscopy without involving mechanical movement.

8.2 Future Directions

The dissertation presents the development and improvement of depth-sensitive spectroscopy with practical strategies, which still have room to improve in biomedical applications. Some challenges need to be resolved to meet the requirements of *in vivo* measurements. These challenges and potential solutions are discussed below:

8.2.1 Lock-in Detection for Raman Measurements

In chapter 5, we demonstrate that lock-in detection can be used to enhance the Raman signal behind scattering media. We use a monochromator to isolate the signal with a specific wavelength and use the signal as feedback for optimization. Then we scan the wavelength of the monochromator to generate the spectrum because the signal has such a high frequency that no commercial spectrometer is fast enough to measure the signal in MHz scale. However, the efficiency of the monochromator is relatively low. While current commercial monochromators can only isolate the signal with a single wavelength, a DMD-based spectrometer, on the other hand, could be the better choice for its fine resolution and ability to measure signals from multiple discrete wavelengths. Since the DMD-based can isolate multiple discrete wavelengths, we can enhance the signal with multiple Raman peaks for a known material instead of a single peak. Also, we can use the Raman peak of water for enhancement for samples merged with a common

background like water. With the spatial filtering technique described in **Chapter 3**, the Raman signals from water and the material dissolved in water from the target location can be enhanced simultaneously. In this way, even without prior knowledge of the sample, it is still possible to enhance the Raman signal of a target. As illustrated in **Fig. 8.1**, several chemicals are mixed in a hydrogel phantom. The Raman peak can be observed for each component along with the hydrogel. Therefore, if we can isolate the Raman peak of hydrogel and use the lock-in method to perform the feedback-based wavefront shaping, the signal from other chemical components can also be enhanced. By using this method, prior knowledge is not required for signal enhancement. Since water is a major component in the tissue, we can use the Raman peak of water as feedback to enhance signals from other unknown components for analysis. Therefore, the future work of this proposed technique is to investigate the performance of Raman enhancement in different biological samples and the possibility of using it for *in vivo* measurements.

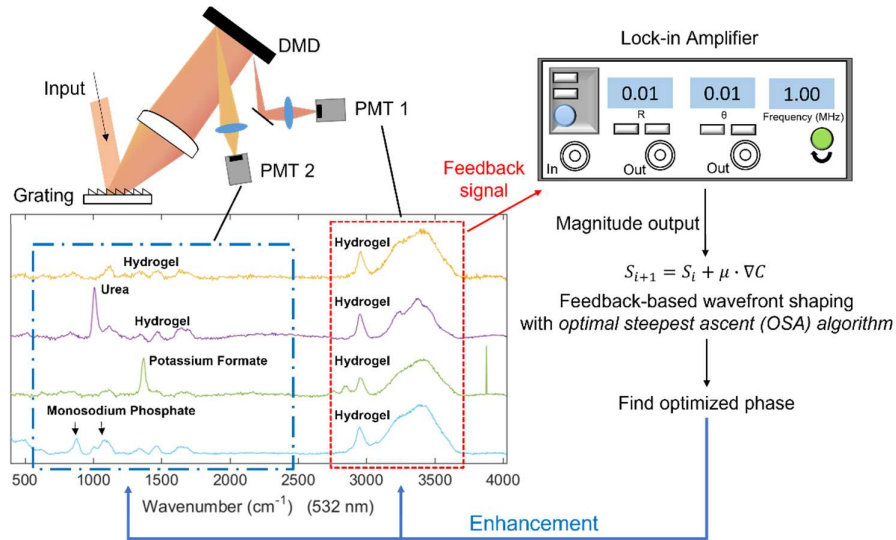


Fig. 8.1. Feedback-based wavefront shaping with lock-in detection for Raman signal enhancement by using common Raman peak as feedback. DMD: Digital micromirror device.

8.2.2 Multi-channel Lock-in Detection for Generating Spatiotemporal Focusing

In **Chapter 5**, we have demonstrated that spatiotemporal focusing can be generated for high enhancement by using the summation of the magnitude of multiple frequencies. Compared to spatial-only focusing, spatiotemporal focusing yields a much stronger focus. Inspired by the high enhancement achieved using the magnitudes of multiple RFs, as shown in **Fig. 5.3 (Chapter 5, section 5.3.1)**, if we can apply lock-in amplification to all beat frequency components, we will be able to generate spatiotemporal focusing with a higher enhancement factor. As a preliminary trial of this idea, we tried using a single-channel lock-in amplifier and scanning the frequency of the driving frequency. We used 32 driving frequencies in this experimental demonstration with a constant spacing of 1 MHz (65, 66 ... 96 MHz). The lock-in amplifier was set to scan the frequency from 1 MHz to 10 MHz and get the magnitude of each frequency sequentially. Then all the magnitudes were added up to serve as feedback. As shown in **Fig. 8.2**, temporal focusing was generated after wavefront shaping using the sequential multi-channel lock-in detection method. However, the optimization took 30 minutes to complete. Therefore, the combination of a multi-channel lock-in amplifier and a summing amplifier is the potential solution in order to speed up the process (**Fig. 8.3**). The temporal focusing with the lock-in method allows it to work with the pulse laser or AOM-modulated light to detect weak signals for higher enhancement. Because AOM-modulated [75] or pulse laser [123] allows laser light to illuminate the sample only within a short time window during temporal focusing, the peak signal from the temporal focusing can be collected without influence from non-focusing signals outside the time

window. Consequently, instead of high-frequency measurements as described in Chapter 5 that require high-bandwidth detectors, commercial spectrometers, and microscopy equipped with low-bandwidth 1D or 2D detectors can be used to measure resulting spectra and images, which is more convenient and practical in clinical research.

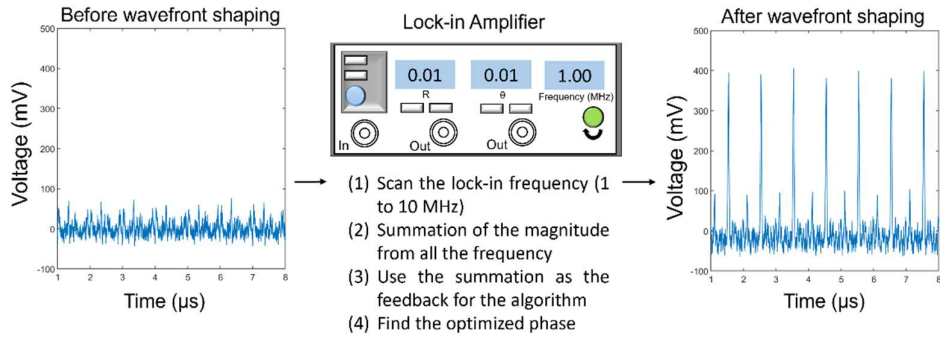


Fig. 8.2. Preliminary study of multi-channel lock-in method for temporal focusing. Sequentially performing lock-in detection of 10 frequency (1, 2...10 MHz) and using the summation of all magnitudes as feedback, temporal focusing is generated after the feedback-based wavefront shaping.

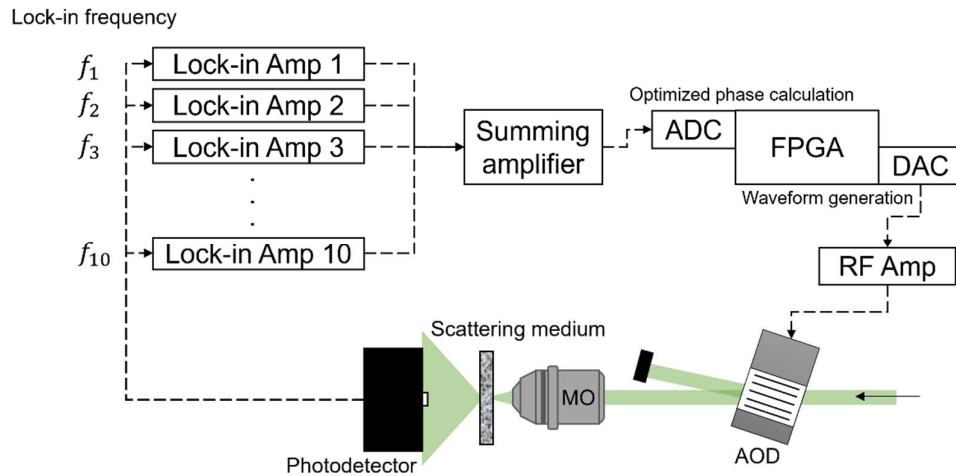


Fig. 8.3. Proposed method for spatiotemporal focusing of a weak signal using multi-channel lock-in amplifier and FPGA acceleration.

8.2.3 FPGA Acceleration for Wavefront Shaping with Lock-in Detection

A critical issue for the lock-in method (**Chapter 5**) is its long optimization time. It takes a few minutes to complete wavefront shaping with lock-in detection in the current configuration. Long optimization time is not suitable for *in vivo* measurements. The critical issue for high-speed wavefront shaping (**Chapter 6**) is the low signal-to-noise ratio for a short data acquisition time. In this case, for weak signals such as fluorescence, the noise could dominate the feedback and result in the poor performance of wavefront shaping. Therefore, building a closed-loop system with a mechanism for selection between two modes, i.e., the mode of lock-in detection for weak signal enhancement and the mode of high-speed wavefront shaping, could utilize the techniques in different conditions (**Fig. 8.4**).

The current setup for **Chapters 5 and 6** could be improved in a few aspects. The first is the slow lock-in amplifier. The bandwidth of the available lock-in amplifier used in the study is up to 200 MHz, but the minimum integration time is as long as 100 μ s. To speed up the process, a more suitable lock-in amplifier with high sensitivity at the specific frequency (1MHz) could detect the feedback with a short integration time. Since the lock-in amplifier provides a DC output (voltage) for the magnitude of the specific frequency, we can capture this DC output with a suitable ADC (low-frequency bandwidth and high resolution in voltage) and use a closed loop to avoid the transfer delay between devices. The proposed method could be used to enhance weak signals like fluorescence and Raman scattering for spatiotemporal focusing with high enhancement in 1 s. The second aspect is to use a stronger pulsed light source for weak signals, such as

fluorescence, w/o *in vivo* measurement. Since time-gating enhancement only needs signal readings in a short time window as feedback, increasing the power in a short time window will increase the signal-to-noise ratio.

Once the system is built, it can be used with different wavefront shaping strategies under various conditions (**Fig. 8.5**).

- (I). Since the feedback is too weak to be detected for extremely weak signals, there will be no enhancement after wavefront shaping, regardless of the strategies used.
- (II). For weak signals such as fluorescence and Raman scattering behind thick static samples, the lock-in method can detect weak signals for spatial focusing with relatively low enhancement. The low signal enhancement method could detect the Raman signal from deep tissues.
- (III). For mild signals, such as fluorescence in thin static samples, band-pass filtering for a particular beat frequency (1MHz in this case) can be used to generate spatial focusing by FPGA. Broad-band filtering (1 to 15 MHz, for instance) can detect spatiotemporal focusing for higher contrast in signal analysis with mild enhancement. The combination could be used to enhance fluorescence signals with a higher enhancement factor than the lock-in method.
- (IV). For strong signals such as laser light measurement, spatiotemporal focusing by using the time-domain signal within a short time window can be used to generate high enhancement for dynamic samples below a dynamic medium due to particle motion, such as blood flow.

The FPGA-based wavefront shaping system will be designed for easier integration with other optical techniques to improve the signal measurement of scattering samples. The future work of this study is to improve and complete the whole system and increase the flexibility of use in different scenarios.

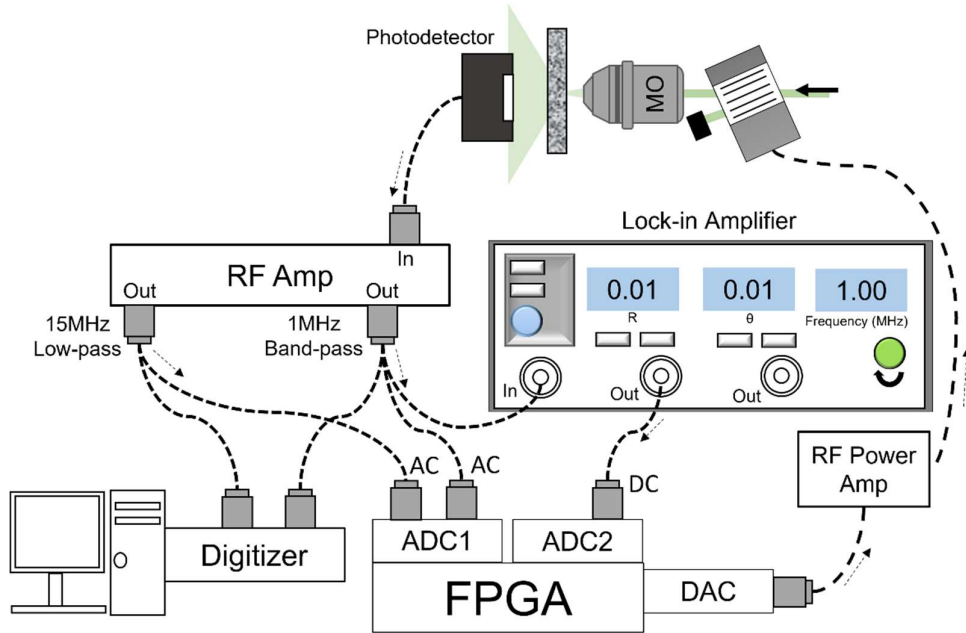


Fig. 8.4. Closed loop system of feedback-based wavefront shaping under different conditions. FPGA: Field-programmable gate array; ADC1: High-bandwidth AC-input analog-to-digital converter; ADC2: High precision DC-input analog-to-digital converter; DAC: Digital-to-analog converter; RF Amp: Broadband (DC – 350 MHz) radiofrequency signal preamplifier.

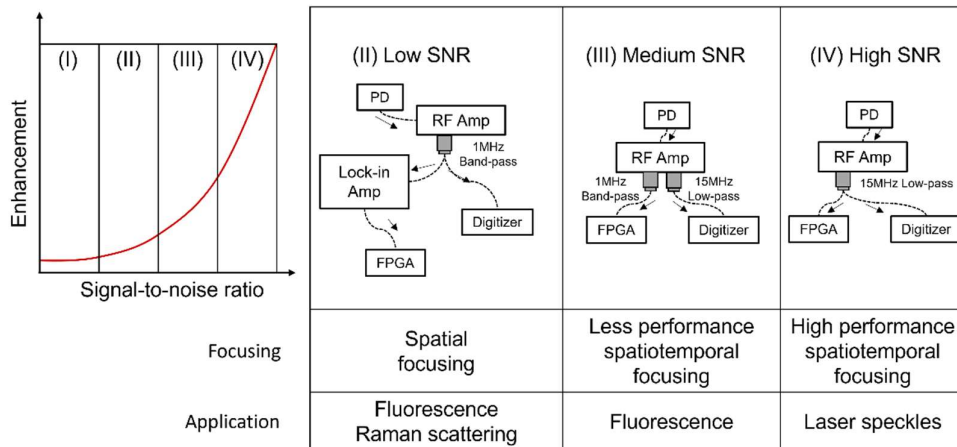


Fig. 8.5. Strategies of wavefront shaping in different conditions based on the SNR of the feedback signal.

Author Publications

Journal (first author)

1. **Hsieh, C. M.**, Malik, M. O., & Liu, Q. (2023). High-speed feedback based wavefront shaping for spatiotemporal enhancement of incoherent light through dynamic scattering media. *Optics Letters*, 48(9), 2313-2316.
2. **Hsieh, C. M.**, Ren, X., & Liu, Q. (2022). Feedback-based wavefront shaping for weak light with lock-in beat frequency detection. *Optics Letters*, 47(19), 5192-5195.
3. **Hsieh, C. M.**, Verma, M., & Liu, Q. (2019). Improving depth sensitive fluorescence spectroscopy with wavefront shaping by spectral and spatial filtering. *IEEE Access*, 7, 170192-170198.

Journal

1. Ren, X., **Hsieh, C. M.**, Malik, M. O., Weiming, J. S., & Liu, Q. (2022). A radio frequency tagging continuous-wave optical spectrometer with megahertz refreshing rate. *IEEE Transactions on Instrumentation and Measurement*.
2. Malik, M. O., Ren, X., **Hsieh, C. M.**, Zhang, Y., & Liu, Q. (2022). Investigation of equivalence between non-resonant Raman Excitation Spectroscopy and conventional Raman Spectroscopy. *IEEE Journal of Selected Topics in Quantum Electronics*, 29(4: Biophotonics), 1-9.
3. Ren, X., Lin, K., **Hsieh, C. M.**, Liu, L., Ge, X., & Liu, Q. (2022). Optical coherence tomography-guided confocal Raman microspectroscopy for rapid measurements in tissues. *Biomedical Optics Express*, 13(1), 344-357.

4. Zhang, Y., Kang, J., **Hsieh, C. M.**, & Liu, Q. (2020). Compressive optical spectrometry based on sequency-ordered Hadamard transform. *IEEE Photonics Journal*, 12(5), 1-8.
5. Ju, J., **Hsieh, C. M.**, Tian, Y., Kang, J., Chia, R., Chang, H., ... & Liu, Q. (2020). Surface enhanced Raman spectroscopy based biosensor with a microneedle array for minimally invasive *in vivo* glucose measurements. *ACS sensors*, 5(6), 1777-1785.

Conference

1. **Chao-Mao Hsieh**, Manish Verma, Quan Liu. (2018, January). Development of Wavefront Shaping for Optical Spectroscopy. Paper presented at Focus on Microscopy
2. **Chao-Mao Hsieh**, Quan Liu. (2019, June). Improving depth sensitive fluorescence spectroscopy with wavefront shaping by spectral and spatial filtering. Paper presented at European Conference on Biomedical Optics (SPIE, OSA).

References

1. Pavlova, I., et al., *Microanatomical and Biochemical Origins of Normal and Precancerous Cervical Autofluorescence Using Laser-scanning Fluorescence Confocal Microscopy*. Photochemistry and photobiology, 2003. **77**(5): p. 550-555.
2. Hanlon, E., et al., *Prospects for in vivo Raman spectroscopy*. Physics in Medicine & Biology, 2000. **45**(2): p. R1.
3. Carlson, J.A., et al., *Molecular diagnostics in melanoma*. Journal of the American Academy of Dermatology, 2005. **52**(5): p. 743-775.
4. Santos, I.s.P., et al., *Raman spectroscopic characterization of melanoma and benign melanocytic lesions suspected of melanoma using high-wavenumber Raman spectroscopy*. Analytical chemistry, 2016. **88**(15): p. 7683-7688.
5. Zhao, J., et al., *Real-time Raman spectroscopy for automatic in vivo skin cancer detection: an independent validation*. Analytical and bioanalytical chemistry, 2015. **407**(27): p. 8373-8379.
6. Liu, Q. and N. Ramanujam, *Experimental proof of the feasibility of using an angled fiber-optic probe for depth-sensitive fluorescence spectroscopy of turbid media*. Optics Letters, 2004. **29**(17): p. 2034-2036.
7. Schwarz, R.A., et al., *Noninvasive evaluation of oral lesions using depth-sensitive optical spectroscopy*. Cancer, 2009. **115**(8): p. 1669-1679.
8. Liu, Q. and N. Ramanujam, *Sequential estimation of optical properties of a two-layered epithelial tissue model from depth-resolved ultraviolet-visible diffuse reflectance spectra*. Applied optics, 2006. **45**(19): p. 4776-4790.
9. Mahadevan-Jansen, A., et al., *Development of a fiber optic probe to measure NIR Raman spectra of cervical tissue in vivo*. Photochemistry and photobiology, 1998. **68**(3): p. 427-431.
10. Matousek, P., *Deep non-invasive Raman spectroscopy of living tissue and powders*. Chemical Society Reviews, 2007. **36**(8): p. 1292-1304.
11. Yu, H., et al., *Recent advances in wavefront shaping techniques for biomedical applications*. Current Applied Physics, 2015. **15**(5): p. 632-641.
12. Schmitt, J.M. and G. Kumar, *Optical scattering properties of soft tissue: a discrete particle model*. Applied optics, 1998. **37**(13): p. 2788-2797.

13. Yaqoob, Z., et al., *Optical phase conjugation for turbidity suppression in biological samples*. Nature photonics, 2008. **2**(2): p. 110-115.
14. Schott, S., et al., *Characterization of the angular memory effect of scattered light in biological tissues*. Optics express, 2015. **23**(10): p. 13505-13516.
15. Ruan, H., et al., *Deep tissue optical focusing and optogenetic modulation with time-reversed ultrasonically encoded light*. Science advances, 2017. **3**(12): p. eaao5520.
16. Morgner, U., et al., *Spectroscopic optical coherence tomography*. Optics letters, 2000. **25**(2): p. 111-113.
17. Dunsby, C. and P. French, *Techniques for depth-resolved imaging through turbid media including coherence-gated imaging*. Journal of Physics D: Applied Physics, 2003. **36**(14): p. R207.
18. Helmchen, F. and W. Denk, *Deep tissue two-photon microscopy*. Nature methods, 2005. **2**(12): p. 932-940.
19. Carminati, R., et al., *Reciprocity, unitarity, and time-reversal symmetry of the S matrix of fields containing evanescent components*. Physical review A, 2000. **62**(1): p. 012712.
20. Kang, S., et al., *Imaging deep within a scattering medium using collective accumulation of single-scattered waves*. Nature Photonics, 2015. **9**(4): p. 253-258.
21. Jeong, S., et al., *Focusing of light energy inside a scattering medium by controlling the time-gated multiple light scattering*. Nature Photonics, 2018. **12**(5): p. 277-283.
22. Kadobianskyi, M., et al., *Scattering correlations of time-gated light*. Optica, 2018. **5**(4): p. 389-394.
23. Selb, J.J., D.K. Joseph, and D.A. Boas, *Time-gated optical system for depth-resolved functional brain imaging*. Journal of biomedical optics, 2006. **11**(4): p. 044008.
24. Patel, N.L., et al., *Relative capacities of time-gated versus continuous-wave imaging to localize tissue embedded vessels with increasing depth*. Journal of Biomedical Optics, 2010. **15**(1): p. 016015.
25. Nissinen, I., et al., *On the effects of the time gate position and width on the signal-to-noise ratio for detection of Raman spectrum in a time-gated CMOS single-photon avalanche diode based sensor*. Sensors and Actuators B: Chemical, 2017. **241**: p. 1145-1152.

26. Gioux, S., A. Mazhar, and D.J. Cuccia, *Spatial frequency domain imaging in 2019: principles, applications, and perspectives*. Journal of biomedical optics, 2019. **24**(7): p. 071613.
27. Cuccia, D.J., et al., *Modulated imaging: quantitative analysis and tomography of turbid media in the spatial-frequency domain*. Optics letters, 2005. **30**(11): p. 1354-1356.
28. Cuccia, D.J., et al., *Quantitation and mapping of tissue optical properties using modulated imaging*. Journal of biomedical optics, 2009. **14**(2): p. 024012.
29. Weber, J.R., et al., *Multispectral imaging of tissue absorption and scattering using spatial frequency domain imaging and a computed-tomography imaging spectrometer*. Journal of biomedical optics, 2011. **16**(1): p. 011015.
30. McClatchy, D.M., et al., *Wide-field quantitative imaging of tissue microstructure using sub-diffuse spatial frequency domain imaging*. Optica, 2016. **3**(6): p. 613-621.
31. Tromberg, B.J., et al., *Non-invasive in vivo characterization of breast tumors using photon migration spectroscopy*. Neoplasia, 2000. **2**(1-2): p. 26-40.
32. Tyson, R.K. and B.W. Frazier, *Principles of adaptive optics*. 2022: CRC press.
33. Bridges, W., et al., *Coherent optical adaptive techniques*. Applied Optics, 1974. **13**(2): p. 291-300.
34. Booth, M.J., *Adaptive optics in microscopy*. Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences, 2007. **365**(1861): p. 2829-2843.
35. Booth, M.J., et al., *Adaptive aberration correction in a confocal microscope*. Proceedings of the National Academy of Sciences, 2002. **99**(9): p. 5788-5792.
36. Tang, J., R.N. Germain, and M. Cui, *Superpenetration optical microscopy by iterative multiphoton adaptive compensation technique*. Proceedings of the National Academy of Sciences, 2012. **109**(22): p. 8434-8439.
37. Qin, Z., et al., *Adaptive optics two-photon microscopy enables near-diffraction-limited and functional retinal imaging in vivo*. Light: Science & Applications, 2020. **9**(1): p. 1-11.
38. Vellekoop, I.M., *Feedback-based wavefront shaping*. Optics express, 2015. **23**(9): p. 12189-12206.

39. Vellekoop, I.M., et al., *Demixing light paths inside disordered metamaterials*. Optics express, 2008. **16**(1): p. 67-80.
40. Mosk, A.P., et al., *Controlling waves in space and time for imaging and focusing in complex media*. Nature photonics, 2012. **6**(5): p. 283-292.
41. Choi, Y., et al., *Overcoming the diffraction limit using multiple light scattering in a highly disordered medium*. Physical review letters, 2011. **107**(2): p. 023902.
42. Bertolotti, J., et al., *Non-invasive imaging through opaque scattering layers*. Nature, 2012. **491**(7423): p. 232-234.
43. Kim, M., et al., *Transmission matrix of a scattering medium and its applications in biophotonics*. Optics express, 2015. **23**(10): p. 12648-12668.
44. Papadopoulos, I.N., et al., *Scattering compensation by focus scanning holographic aberration probing (F-SHARP)*. Nature Photonics, 2017. **11**(2): p. 116-123.
45. Vellekoop, I.M. and C.M. Aegerter, *Scattered light fluorescence microscopy: imaging through turbid layers*. Optics letters, 2010. **35**(8): p. 1245-1247.
46. Katz, O., et al., *Noninvasive nonlinear focusing and imaging through strongly scattering turbid layers*. Optica, 2014. **1**(3): p. 170-174.
47. Thompson, J.V., et al., *Enhanced second harmonic generation efficiency via wavefront shaping*. ACS Photonics, 2017. **4**(7): p. 1790-1796.
48. Liu, Y., et al., *Focusing light inside dynamic scattering media with millisecond digital optical phase conjugation*. Optica, 2017. **4**(2): p. 280-288.
49. Wang, D., et al., *Focusing through dynamic tissue with millisecond digital optical phase conjugation*. Optica, 2015. **2**(8): p. 728-735.
50. Hemphill, A.S., et al., *High-speed single-shot optical focusing through dynamic scattering media with full-phase wavefront shaping*. Applied Physics Letters, 2017. **111**(22): p. 221109.
51. Popoff, S.M., et al., *Measuring the transmission matrix in optics: an approach to the study and control of light propagation in disordered media*. Physical review letters, 2010. **104**(10): p. 100601.
52. Yoon, J., et al., *Measuring optical transmission matrices by wavefront shaping*. Optics Express, 2015. **23**(8): p. 10158-10167.

53. Blochet, B., L. Bourdieu, and S. Gigan, *Focusing light through dynamical samples using fast continuous wavefront optimization*. Optics letters, 2017. **42**(23): p. 4994-4997.
54. Tzang, O., et al., *Wavefront shaping in complex media with a 350 kHz modulator via a 1D-to-2D transform*. Nature Photonics, 2019. **13**(11): p. 788-793.
55. Freund, I., *Looking through walls and around corners*. Physica A: Statistical Mechanics and its Applications, 1990. **168**(1): p. 49-65.
56. Vellekoop, I.M. and A. Mosk, *Focusing coherent light through opaque strongly scattering media*. Optics letters, 2007. **32**(16): p. 2309-2311.
57. Vellekoop, I.M., *Controlling the propagation of light in disordered scattering media*. arXiv preprint arXiv:0807.1087, 2008.
58. Tzang, O., et al., *Adaptive wavefront shaping for controlling nonlinear multimode interactions in optical fibres*. Nature Photonics, 2018. **12**(6): p. 368-374.
59. Vellekoop, I.M. and A. Mosk, *Universal optimal transmission of light through disordered materials*. Physical review letters, 2008. **101**(12): p. 120601.
60. van Putten, E.G., et al., *Scattering lens resolves sub-100 nm structures with visible light*. Physical review letters, 2011. **106**(19): p. 193905.
61. Vellekoop, I.M., A. Lagendijk, and A. Mosk, *Exploiting disorder for perfect focusing*. Nature photonics, 2010. **4**(5): p. 320-322.
62. Kohlgraf-Owens, T.W. and A. Dogariu, *Transmission matrices of random media: means for spectral polarimetric measurements*. Optics letters, 2010. **35**(13): p. 2236-2238.
63. Mounaix, M., et al., *Spatiotemporal coherent control of light through a multiple scattering medium with the multispectral transmission matrix*. Physical review letters, 2016. **116**(25): p. 253901.
64. Osnabrugge, G., et al., *Generalized optical memory effect*. Optica, 2017. **4**(8): p. 886-892.
65. Conkey, D.B., A.M. Caravaca-Aguirre, and R. Piestun, *High-speed scattering medium characterization with application to focusing light through turbid media*. Optics express, 2012. **20**(2): p. 1733-1740.
66. Akbulut, D., et al., *Focusing light through random photonic media by binary amplitude modulation*. Optics express, 2011. **19**(5): p. 4017-4029.

67. Turtaev, S., et al., *Comparison of nematic liquid-crystal and DMD based spatial light modulation in complex photonics*. Optics express, 2017. **25**(24): p. 29874-29884.
68. Yu, H., K. Lee, and Y. Park, *Ultrahigh enhancement of light focusing through disordered media controlled by mega-pixel modes*. Optics express, 2017. **25**(7): p. 8036-8047.
69. Xu, X., H. Liu, and L.V. Wang, *Time-reversed ultrasonically encoded optical focusing into scattering media*. Nature photonics, 2011. **5**(3): p. 154-157.
70. Liu, Y., et al., *Optical focusing deep inside dynamic scattering media with near-infrared time-reversed ultrasonically encoded (TRUE) light*. Nature communications, 2015. **6**(1): p. 1-9.
71. Judkewitz, B., et al., *Speckle-scale focusing in the diffusive regime with time reversal of variance-encoded light (TROVE)*. Nature photonics, 2013. **7**(4): p. 300-305.
72. Ruan, H., M. Jang, and C. Yang, *Optical focusing inside scattering media with time-reversed ultrasound microbubble encoded light*. Nature communications, 2015. **6**(1): p. 1-8.
73. Badon, A., et al., *Smart optical coherence tomography for ultra-deep imaging through highly scattering media*. Science advances, 2016. **2**(11): p. e1600370.
74. Zhou, E.H., et al., *Focusing on moving targets through scattering samples*. Optica, 2014. **1**(4): p. 227-232.
75. Feldkhun, D., et al., *Focusing and scanning through scattering media in microseconds*. Optica, 2019. **6**(1): p. 72-75.
76. Wei, X., et al., *Real-time frequency-encoded spatiotemporal focusing through scattering media using a programmable 2D ultrafine optical frequency comb*. Science advances, 2020. **6**(8): p. eaay1192.
77. Horstmeyer, R., H. Ruan, and C. Yang, *Guidestar-assisted wavefront-shaping methods for focusing light into biological tissue*. Nature photonics, 2015. **9**(9): p. 563-571.
78. Li, D., et al., *Non-invasive optical focusing inside strongly scattering media with linear fluorescence*. Applied Physics Letters, 2020. **116**(24): p. 241104.
79. Thompson, J.V., et al., *Wavefront shaping enhanced Raman scattering in a turbid medium*. Optics letters, 2016. **41**(8): p. 1769-1772.

80. Kong, F., et al., *Photoacoustic-guided convergence of light through optically diffusive media*. Optics letters, 2011. **36**(11): p. 2053-2055.
81. Ruan, H., et al., *Focusing light inside scattering media with magnetic-particle-guided wavefront shaping*. Optica, 2017. **4**(11): p. 1337-1343.
82. Lai, P., et al., *Photoacoustically guided wavefront shaping for enhanced optical focusing in scattering media*. Nature photonics, 2015. **9**(2): p. 126-132.
83. Fenno, L., O. Yizhar, and K. Deisseroth, *The development and application of optogenetics*. Annual review of neuroscience, 2011. **34**: p. 389.
84. Deisseroth, K., *Optogenetics*. Nature methods, 2011. **8**(1): p. 26-29.
85. Cui, M., *Parallel wavefront optimization method for focusing light through random scattering media*. Optics letters, 2011. **36**(6): p. 870-872.
86. Yoon, J., et al., *Optogenetic control of cell signaling pathway through scattering skull using wavefront shaping*. Scientific reports, 2015. **5**(1): p. 1-7.
87. Bañas, A. and J. Glückstad, *Holo-GPC: holographic generalized phase contrast*. Optics Communications, 2017. **392**: p. 190-195.
88. Accanto, N., et al., *Multiplexed temporally focused light shaping for high-resolution multi-cell targeting*. Optica, 2018. **5**(11): p. 1478-1491.
89. Dholakia, K. and T. Čižmár, *Shaping the future of manipulation*. Nature photonics, 2011. **5**(6): p. 335-342.
90. Čižmár, T., M. Mazilu, and K. Dholakia, *In situ wavefront correction and its application to micromanipulation*. Nature Photonics, 2010. **4**(6): p. 388-394.
91. Rueckel, M., J.A. Mack-Bucher, and W. Denk, *Adaptive wavefront correction in two-photon microscopy using coherence-gated wavefront sensing*. Proceedings of the National Academy of Sciences, 2006. **103**(46): p. 17137-17142.
92. Yu, H., et al., *In vivo deep tissue imaging using wavefront shaping optical coherence tomography*. Journal of biomedical optics, 2016. **21**(10): p. 101406.
93. Yu, H., et al., *Depth-enhanced 2-D optical coherence tomography using complex wavefront shaping*. Optics express, 2014. **22**(7): p. 7514-7523.

94. Kanngiesser, J., M. Rahlves, and B. Roth, *Iterative wavefront correction for complex spectral domain optical coherence tomography*. Optics Letters, 2019. **44**(6): p. 1347-1350.
95. Bertolotti, J. and O. Katz, *Imaging in complex media*. Nature Physics, 2022. **18**(9): p. 1008-1017.
96. Vasquez-Lopez, S.A., et al., *Subcellular spatial resolution achieved for deep-brain imaging in vivo using a minimally invasive multimode fiber*. Light: Science & Applications, 2018. **7**(1): p. 1-6.
97. Park, J.-H., W. Sun, and M. Cui, *High-resolution in vivo imaging of mouse brain through the intact skull*. Proceedings of the National Academy of Sciences, 2015. **112**(30): p. 9236-9241.
98. Arias, A. and P. Artal, *Wavefront-shaping-based correction of optically simulated cataracts*. Optica, 2020. **7**(1): p. 22-27.
99. Vellekoop, I.M., M. Cui, and C. Yang, *Digital optical phase conjugation of fluorescence in turbid tissue*. Applied physics letters, 2012. **101**(8): p. 081108.
100. Nixon, M., et al., *Real-time wavefront shaping through scattering media by all-optical feedback*. Nature Photonics, 2013. **7**(11): p. 919-924.
101. Fayyaz, Z., et al. *Wavefront shaping using simulated annealing algorithm for focusing light through turbid media*. in *Photons Plus Ultrasound: Imaging and Sensing 2018*. 2018. SPIE.
102. Conkey, D.B., et al., *Genetic algorithm optimization for focusing through turbid media in noisy environments*. Optics express, 2012. **20**(5): p. 4840-4849.
103. Vellekoop, I.M. and A. Mosk, *Phase control algorithms for focusing light through turbid media*. Optics communications, 2008. **281**(11): p. 3071-3080.
104. Verma, M. and Q. Liu. *Evaluation of the optimal steepest ascent algorithm for focusing light through turbid media*. in *Optical Coherence Imaging Techniques and Imaging in Scattering Media II*. 2017. Munich: Optica Publishing Group.
105. Greening, G.J., et al., *Characterization of thin poly (dimethylsiloxane)-based tissue-simulating phantoms with tunable reduced scattering and absorption coefficients at visible and near-infrared wavelengths*. Journal of Biomedical Optics, 2014. **19**(11): p. 115002.
106. Mätzler, C., *MATLAB functions for Mie scattering and absorption, version 2*. 2002.

107. Zhu, C. and Q. Liu, *Validity of the semi-infinite tumor model in diffuse reflectance spectroscopy for epithelial cancer diagnosis: a Monte Carlo study*. Optics Express, 2011. **19**(18): p. 17799-17812.
108. Purnapatra, S.B., S. Bera, and P.P. Mondal, *Spatial filter based Bessel-like beam for improved penetration depth imaging in fluorescence microscopy*. Scientific reports, 2012. **2**(1): p. 1-11.
109. Ramanujam, N., *Fluorescence spectroscopy of neoplastic and non-neoplastic tissues*. Neoplasia, 2000. **2**(1-2): p. 89-117.
110. Ramanujam, N., *Fluorescence spectroscopy in vivo*. Encyclopedia of analytical chemistry, 2000. **1**: p. 20-56.
111. Andersen, T.E., et al., *Novel concept for large deformable mirrors*. Optical Engineering, 2006. **45**(7): p. 073001.
112. Hosaka, S., et al., *High speed laser beam scanning using an acousto-optical deflector (AOD)*. Japanese journal of applied physics, 1987. **26**(7R): p. 1026.
113. Kaplan, A., N. Friedman, and N. Davidson, *Acousto-optic lens with very fast focus scanning*. Optics letters, 2001. **26**(14): p. 1078-1080.
114. Reddy, G.D. and P. Saggau, *Fast three-dimensional laser scanning scheme using acousto-optic deflectors*. Journal of biomedical optics, 2005. **10**(6): p. 064038.
115. Griffiths, V.A., et al., *Real-time 3D movement correction for two-photon imaging in behaving animals*. Nature methods, 2020. **17**(7): p. 741-748.
116. Salomé, R., et al., *Ultrafast random-access scanning in two-photon microscopy using acousto-optic deflectors*. Journal of neuroscience methods, 2006. **154**(1-2): p. 161-174.
117. Moffitt, J.R., et al., *Recent advances in optical tweezers*. Annu. Rev. Biochem., 2008. **77**: p. 205-228.
118. Vermeulen, K.C., et al., *Calibrating bead displacements in optical tweezers using acousto-optic deflectors*. Review of Scientific Instruments, 2006. **77**(1): p. 013704.
119. Kirkby, P.A., K.N.S. Nadella, and R.A. Silver, *A compact acousto-optic lens for 2D and 3D femtosecond based 2-photon microscopy*. Optics express, 2010. **18**(13): p. 13720-13744.
120. Iyer, V., T.M. Hoogland, and P. Saggau, *Fast functional imaging of single neurons using random-access multiphoton (RAMP) microscopy*. Journal of neurophysiology, 2006. **95**(1): p. 535-545.

121. Iyer, V., B.E. Losavio, and P. Saggau, *Compensation of spatial and temporal dispersion for acousto-optic multiphoton laser-scanning microscopy*. Journal of biomedical optics, 2003. **8**(3): p. 460-471.
122. Konstantinou, G., et al., *Dynamic wavefront shaping with an acousto-optic lens for laser scanning microscopy*. Optics express, 2016. **24**(6): p. 6283-6299.
123. Akemann, W., et al., *Fast spatial beam shaping by acousto-optic diffraction for 3D non-linear microscopy*. Optics express, 2015. **23**(22): p. 28191-28205.
124. Chang, I., *Acousto-optic devices and applications*. Handbook of optics, 1995. **2**: p. 12.1-12.54.
125. Rivas, J.G., et al., *Optical transmission through strong scattering and highly polydisperse media*. EPL (Europhysics Letters), 1999. **48**(1): p. 22.
126. Hsieh, C.-M., M. Verma, and Q. Liu, *Improving depth sensitive fluorescence spectroscopy with wavefront shaping by spectral and spatial filtering*. IEEE Access, 2019. **7**: p. 170192-170198.
127. Marriott, G., et al., *Optical lock-in detection imaging microscopy for contrast-enhanced imaging in living cells*. Proceedings of the National Academy of Sciences, 2008. **105**(46): p. 17789-17794.
128. Tzang, O. and R. Piestun, *Lock-in detection of photoacoustic feedback signal for focusing through scattering media using wave-front shaping*. Optics Express, 2016. **24**(24): p. 28122-28130.
129. Picqué, N. and T.W. Hänsch, *Frequency comb spectroscopy*. Nature Photonics, 2019. **13**(3): p. 146-157.
130. Diebold, E.D., et al., *Digitally synthesized beat frequency multiplexing for sub-millisecond fluorescence microscopy*. Nature Photonics, 2013. **7**(10): p. 806-810.
131. Katz, O., et al., *Focusing and compression of ultrashort pulses through scattering media*. Nature photonics, 2011. **5**(6): p. 372-377.
132. Ntziachristos, V., *Going deeper than microscopy: the optical imaging frontier in biology*. Nature methods, 2010. **7**(8): p. 603-614.
133. Hsieh, C.-M., X. Ren, and Q. Liu, *Feedback-based wavefront shaping for weak light with lock-in beat frequency detection*. Optics Letters, 2022. **47**(19): p. 5192-5195.
134. Conkey, D.B., et al. *High-speed phase-control of wavefronts with binary amplitude DMD for light control through dynamic turbid media*. in *MEMS Adaptive Optics VII*. 2013. SPIE.

135. McCabe, D.J., et al., *Spatio-temporal focusing of an ultrafast pulse through a multiply scattering medium*. Nature communications, 2011. **2**(1): p. 1-5.
136. Drezek, R.A., et al., *Understanding the contributions of NADH and collagen to cervical tissue fluorescence spectra: modeling, measurements, and implications*. Journal of biomedical optics, 2001. **6**(4): p. 385-396.
137. Collier, T., et al., *Determination of epithelial tissue scattering coefficient using confocal microscopy*. IEEE Journal of selected topics in quantum electronics, 2003. **9**(2): p. 307-313.
138. Huang, Z., et al., *Laser-induced autofluorescence microscopy of normal and tumor human colonic tissue*. International journal of oncology, 2004. **24**(1): p. 59-63.
139. Drezek, R., et al., *Autofluorescence microscopy of fresh cervical-tissue sections reveals alterations in tissue biochemistry with dysplasia*. Photochemistry and photobiology, 2001. **73**(6): p. 636-641.
140. Zhu, C., Q. Liu, and N. Ramanujam, *Effect of fiber optic probe geometry on depth-resolved fluorescence measurements from epithelial tissues: a Monte Carlo simulation*. Journal of Biomedical Optics, 2003. **8**(2): p. 237-247.
141. Wu, Y., et al., *Depth-resolved fluorescence spectroscopy of normal and dysplastic cervical tissue*. Optics express, 2005. **13**(2): p. 382-388.
142. Polglase, A.L., et al., *A fluorescence confocal endomicroscope for in vivo microscopy of the upper-and the lower-GI tract*. Gastrointestinal endoscopy, 2005. **62**(5): p. 686-695.
143. Scanziani, M. and M. Häusser, *Electrophysiology in the age of light*. Nature, 2009. **461**(7266): p. 930-939.
144. Badon, A., et al., *Video-rate large-scale imaging with Multi-Z confocal microscopy*. Optica, 2019. **6**(4): p. 389-395.
145. Duocastella, M., et al., *Fast inertia-free volumetric light-sheet microscope*. ACS Photonics, 2017. **4**(7): p. 1797-1804.
146. Chan, J.C., et al., *Digitally synthesized beat frequency-multiplexed fluorescence lifetime spectroscopy*. Biomedical Optics Express, 2014. **5**(12): p. 4428-4436.
147. Mikami, H., et al., *Ultrafast confocal fluorescence microscopy beyond the fluorescence lifetime limit*. Optica, 2018. **5**(2): p. 117-126.

148. Kanno, H., et al., *Simple, stable, compact implementation of frequency-division-multiplexed microscopy by inline interferometry*. Optics Letters, 2019. **44**(3): p. 467-470.
149. Zong, W., *New solution for fast axial scanning in fluorescence microscopy*. Light, Science & Applications, 2020. **9**.
150. Chakraborty, T., et al., *Converting lateral scanning into axial focusing to speed up three-dimensional microscopy*. Light: Science & Applications, 2020. **9**(1): p. 1-12.