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2020

Li, D., Sahoo, S. K., Lam, H. Q., Wang, D., & Dang, C. (2020). Non-invasive optical focusing inside strongly scattering media with linear fluorescence. *Applied Physics Letters*, 116(24), 241104-. doi:10.1063/5.0004071

<https://hdl.handle.net/10356/142188>

<https://doi.org/10.1063/5.0004071>

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Non-invasive optical focusing inside strongly scattering media with linear fluorescence

Dayan Li,¹ Sujit Kumar Sahoo,² Huy Quoc Lam,^{1,3} Dong Wang,^{1,4} Cuong Dang,^{1,*}

¹Centre for OptoElectronics and Biophotonics (COEB), School of Electrical and Electronic Engineering, The Photonics Institute (TPI), Nanyang Technological University Singapore, 50 Nanyang Avenue, Singapore 639798, Singapore

²School of Electrical Sciences, Indian Institute of Technology Goa, Goa 403401, India

³Temasek Laboratories @ Nanyang Technological University, 50 Nanyang Avenue, 639798, Singapore

⁴Key Laboratory of Advanced Transducers and Intelligent Control System, Ministry of Education, and Shanxi Province, College of Physics and Optoelectronics, Taiyuan University of Technology, Taiyuan 030024, China

*Corresponding author: hcdang@ntu.edu.sg

Non-invasive optical focusing inside scattering media is still a big challenge because inhomogeneous media scatter both incoming photons for focusing and outgoing photons for observation. Various approaches, utilizing non-linear fluorescence or ultrasound, have been reported to address this difficulty. However, implementation of these methods is complicated and highly expensive, as ultrafast laser systems or photo-acoustic equipment must be employed. Here, we demonstrate a wavefront shaping technique to achieve non-invasive focusing (NiF) inside scattering media using only a linear fluorescent signal. Contrast and mean of incoherent speckles, produced by the linear fluorescence, are utilized as feedback signals to optimize the input wavefront. While increasing speckle contrast makes the focus tighter, and increasing the speckle mean enhances the intensity, fine-tuning the contribution of these two factors in our two-step optimization is essential. An optimal wavefront is found to achieve simultaneously both a micrometer focal spot size (down to 20 μm diameter) and high intensity (more than a 100-fold enhancement) inside the scattering media. Our method promises a route in life science towards focusing, imaging or manipulating deep into biological tissues with linear fluorescent agents.

Optical scattering poses major limits with respect to imaging resolution and depth when modern microscopic techniques are applied to complex media such as biological tissues. The inhomogeneous refractive index of complex media strongly perturbs light propagation and thus prohibits the creation of diffraction-limited focus. While appearing random, light scattering in static media is a deterministic process, and therefore its effect can be 'canceled' or reversed [1, 2]. One can implement wavefront shaping techniques to achieve focusing through scattering media with direct feedback from internal sensors, i.e. invasive approaches [1, 3, 4]. However, in scenarios where direct access to the target plane is not possible [5], the problem becomes much more challenging. Such non-invasive approaches are much more attractive for many medical and biological applications.

Various non-invasive methods (see ref. 6 and references therein) have been proposed to tackle light scattering issues and then focus light into complex media. One can use acoustic waves to eliminate the scattering effect and then non-invasively access the target plane in a photoacoustic feedback approach. The acoustically focusing spot defined by the resolution of the ultrasound wave is the 'guide-star' for wavefront shaping [7-10]. Fully optical approaches usually employ non-linear fluorescence [11, 12] (e.g. the two-photon or multi-photon effect). Because photo-acoustic coupling or non-linear excitation is involved, these methods are subject to the drawbacks of complication and cost in implementation. Using time-gating in coherent systems also helps non-invasive wavefront corrections [13]. When combined with random scattering matrix [14], time gating shows effectiveness in non-invasive focusing [15] or imaging [16, 17] deep inside scattering media. However, these methods [15-17] are preferably being applied to discrete objects; a limitation which is related to the feasibility of implementing 'iterative time reversal' [18, 19]. Recently, non-invasive focusing using speckle correlations has been proposed [20]. Because this

approach is based on the optical memory effect, it is only applicable to relative thin samples and the depth of focus is shallow.

Here, utilizing linear fluorescence as an optical feedback signal, we demonstrate an iterative wavefront shaping technique that is able to non-invasively focus light onto an extended object behind strongly scattering media. The statistical properties of the fluorescence-generated speckle pattern, such as its contrast and mean, are shown respectively related to the focal spot size and intensity enhancement in creating a focus. Because the speckle pattern at the imaging sensor is the incoherent summation of multiple speckle patterns generated by each individual fluorescent source, more sources will reduce the speckle contrast. Therefore, maximizing speckle contrast will concentrate the excitation light onto a single fluorescent source. On the other hand, mean intensity of speckle pattern relates to the amount of excitation light that falls onto the fluorescent sample. By increasing speckle variance (the product of mean intensity and speckle contrast) in optimization, Gigan and co-workers achieved non-invasive focusing on scattered fluorescent beads [21]. In fact, the approach showed the dominance of mean intensity in optimization, and therefore, quickly achieved a loose focus region when the number of discrete fluorescent bead increased. In contrast to this, we implement a two-step optimization approach with controllable contributions of mean intensity and speckle contrast. We start with a mean dominant optimization, followed by a contrast dominant optimization to achieve a tight focal spot with high intensity on a continuously extended object. An optimized wavefront is found to create a focal spot of 20 μm diameter behind the scattering media, while its intensity is enhanced up to 100-fold. Our results demonstrate a major step to control light through strongly scattering media, promising various applications in life science such as optical surgery, drug delivery, and imaging.

A schematic of the non-invasive focusing inside scattering media, as well as a numerical example, are presented in Fig. 1. Input

laser light E_i is scattered before hitting a fluorescent object inside the scattering media. Due to multiple scattering, only a small portion of E_i can reach to the depth of the object. We illustrate this situation in Fig. 1a, where E_e denotes fluorescence excitation light, E_r is the portion of light scattered forward but not reaching to the object and E_b is backward scattered light. The purpose of our non-invasive focusing is to minimize E_r and E_b , thus E_e is maximized, and to further squeeze E_e into a single focal spot. The fluorescent sample absorbs the excitation light coming to it then emits fluorescent light at longer wavelength. The imaging sensor detects the scattered fluorescent light after passing through the scattering media. To achieve a focusing spot on the sample, the input wavefront of E_i must be shaped to overcome the effect of multiple scattering for excitation light while the fluorescent speckle pattern at the imaging sensor is the feedback signal. Here, we propose to use wavefront shaping based on genetic algorithm (GA) to achieve the goal, while other optimizing algorithms are also available[22].

Fig. 1. Non-invasive optical focusing inside scattering media: concept and numerical demonstration. (a) Schematic of non-invasive focusing (NiF) inside scattering media by wavefront shaping. E_i denotes input coherent light and it is multiply scattered into E_r , E_b and E_e ; S_k denotes speckle at detector generated from single k^{th} fluorescent emitting source. Input wavefront is optimized by a GA with feedback from speckle statistics at the detector. (b) Illustrating results of non-invasive focusing from simulation: intensity profiles of fluorescent light at fluorescence plane corresponding to c are shown. (c) Wave propagation simulated in x - z plane, including both forward excitation light and backward fluorescent emission, for random input wavefront (top) and optimized input wavefronts from mean dominant NiF (middle) and two-step NiF (bottom), respectively.

On the object plane, assuming that a fluorescent object can be sampled into K sources. For the single k^{th} source, it emits fluorescent light and, after multiple scattering, generates a speckle pattern $S_k(x)$ at position x on the detector plane. Then $S_k(x)$ is point spread function (PSF) describing fluorescent light propagating from the k^{th} source to detector plane. Unlike the approach relied on memory effect where the PSF is shift-invariant [23, 24], our PSF of $S_k(x)$ can be source dependent, i.e. no memory effect requirement. This can significantly enhance the depth of focusing. The total intensity $I(x)$ at the detector is an incoherent sum of all the speckles generated by each individual source. As a result, $I(x)$ at detector is given by:

$$I(x) = \beta \sum_{k=1}^K |E_e^{(k)}|^2 S_k(x), \quad (1)$$

where $E_e^{(k)}$ is the excitation light field that illuminates the k^{th} source. Because it is a linear fluorescence, the response of fluorescence is proportional to excitation intensity rather than the field of illuminating light. β denotes fluorescence's photon conversion efficiency.

From Eq. (1) and theory of speckle statistics [25], we know that contrast of $I(x)$ pattern, $C(I)$, is inversely proportional to $\sqrt{K}$. As a result, if one can optimize the input wavefront such that fewer fluorescent areas are excited, the contrast $C(I)$ will be increased. In principle, when only a single source is excited, i.e. creating a focus, $C(I)$ reaches its maximum value. We also see from Eq. (1) that total intensity of $I(x)$ is linearly proportional to the mean value of illuminating intensity on the fluorescent object. In order to create a focus with higher intensity enhancement, one also needs to optimize for $M(I)$, the mean value of $I(x)$.

Here, we can define a flexible cost function, $F(I) = \alpha * C(I) + M(I)$, as the feedback signal for GA, where α is a constant controlled by users. When $\alpha * C(I) \ll M(I)$, GA implements a mean dominant NiF. Similarly, when $\alpha * C(I) \gg M(I)$, a contrast dominant NiF will be carried out. By controlling α , we can fine-tune the optimization approach in a broad range from mean dominant NiF to contrast dominant NiF to achieve the goal. Mean dominant NiF tends to increase amount of excitation light onto the fluorescence area, which might lead to a loose focusing spot or multiple spots (loose focusing). On the other hand, subsequent contrast dominant NiF promotes a shrinking size of excitation spot on the fluorescence sample, leading to a tighter focus spot; however, intensity enhancement is ultimately limited.

As a numerical example, the system depicted in Fig. 1a is simulated. Thanks to μ -diff [26], an open-source Matlab toolbox for solving the multiple scattering problem, scattered light fields inside two dimensional (x - z plane) scattering media can be easily calculated. The forward propagation of coherent excitation light and backward propagation of fluorescent emission light, as well as GA based wavefront shaping, are all simulated and integrated together, so that non-invasive focusing is numerically investigated. Details about the simulation method can be found in [supplementary material].

Fig. 1b shows the intensity profiles at the fluorescent object plane for different input wavefronts. We see that at the initial condition, when a random wavefront is illuminating the scattering media, fluorescent intensity is low and almost flat, implying the multiple scattering effect imposed to the input light. Fig. 1c top illustrates this situation. We see that input light is scattered out of the scattering media and only a small portion of light can reach the

fluorescent object. In return, only small amount of fluorescent emission is observed. In Fig.1 c top, we also observed obvious light interference arising from coherent waves propagating in the scattering media, while the incoherent fluorescent emission light does not have such effect. After wavefront optimization with mean dominant cost function, we indeed see a significant increase of fluorescent intensity (blue curve in Fig. 1b). This corresponds to Fig. 1c middle. As can be seen, a very large portion of input light is guided onto the fluorescent object, which implies that E_e is enhanced by mean dominant NiF. However, at this stage, only a loose focus is obtained; which is similar to optimization with speckle variance as the cost function [21]. Continuing the wavefront optimization process, but now switching to contrast dominant NiF, finally we obtain a tighter focus, with intensity at the focal spot further increased (Fig. 1b red curve). From Fig. 1c bottom, we clearly see that more E_e is squeezed onto a reduced fluorescent area.

Now we turn to experimental verification of the proposed NiF concept. Details regarding to experimental setup and sample preparation can be found in [supplementary material]. Fig. 2 presents the focus development during the iterations of wavefront shaping, our proof of concept demonstration with experiments. During the wavefront optimization process, we first carry out mean dominant NiF, by choosing the α value such that the cost function $F(I) = \alpha * C(I) + M(I)$ is dominated by mean value. For example, here we set $\alpha = 170$, so that the initial ratio of $\alpha * C(I)$ to $M(I)$ is 0.1; see Fig. 2(a) left axis. It is observed that the cost function value continues to increase until iteration 320, when it then reaches saturation. If we compare the object image at iteration 320 (Fig. 2d) to that at initial condition (Fig. 2c), it is obvious that a considerable amount of light has been directed to the fluorescent object. By summing up the intensity values for the pixels on the object area, we estimate that fluorescent light intensity has been enhanced by 12.12 times after these iterations. However, despite remarkable intensity enhancement, the optimization only achieves a slight focusing effect. This result is understandable as enhancing the mean of a linear signal is equivalent to increasing the excitation light on the fluorescent sample.

Fig. 2. Experimental results of focus development. (a) Plot of the cost function value at each iteration during optimizations. Left axis, mean dominant NiF; right axis, contrast dominant NiF. (b) Plot of focal spot size and intensity enhancement at position of focus over iterations. Images on object plane at some typical iterations: (c) before optimization, (d) at iteration 320, (e) at iteration 530, (f) at iteration 620. The intensity in the captured images (8 bits) is normalized with the exposure time to

display. Inset: intensity profile of focal spot through dashed blue line in (f). Scale bar: 32 μm .

In Fig. 2b, we also plot the focal spot size and intensity enhancement factor at the focal point over different optimization iterations. The focal spot size is calculated by counting the number of pixels that have intensity values greater than 10% of maximum intensity at each object image. The calculated spot size at each iteration is normalized by the spot size calculated from the object image at initial condition. As for the intensity enhancement, the value is calculated as the ratio of the focal point intensity at each iteration to the corresponding value at the initial condition. From Fig. 2b, we see that over the first 320 iterations, while intensity at the focal point is continually enhanced (~ 45 -fold), the focal spot size is hardly reduced. This observation agrees well with our previous observation in simulation and our intuition that mean dominant NiF mainly optimizes intensity at fluorescent object. Some small contribution from speckle contrast in cost function is insufficient to create a tight focal spot.

After gaining a strong fluorescent signal with mean dominant NiF, we switch the optimization to contrast dominant NiF at the basis of iteration 320. Here, we set $\alpha = 17000$, where now the ratio of $\alpha * C(I)$ to $M(I)$ is 60. From Fig. 2a right axis, we see that the cost function value increases during optimizations and reaches saturation after about 620 iterations. Interestingly, the blue curve in Fig. 2b and object images in Fig. 2e and Fig. 2f show that a focal spot is gradually created during the contrast dominant NiF process. For example, comparing the object image at iteration 520 (Fig. 2e) to that at 320 (Fig. 2d), we visually observe the shrinking of object size. More importantly, we see from Fig. 2b that the intensity at the focal point continues increasing, while its size is reducing by contrast dominant NiF. Finally, at iteration 620 (Fig. 2f), a focal spot is created with saturation of the contrast dominant cost function. Thanks to our two-step optimization, intensity at focal spot has achieved about 100-fold enhancement compared to the initial object (red curve in Fig. 2b). The focal spot size is Gaussian with measured FWHM of 20 μm , as shown in the inset on Fig. 2f.

Now, we examine the speckle evolution over the iterations of wavefront shaping as presented in Fig. 3. The first observation is that the speckle area is shrinking over iterations (Fig. 3a-c). This is good indication that the fluorescent area at the object plane is also reducing. During the first stage of optimization, i.e. mean dominant NiF, the speckle's mean intensity increases as expected. Also, the standard deviation, which is essentially dominated by speckle mean, increases as well. After switching to contrast dominant NiF, both mean and standard deviation decreases (Fig. 3d and f). More importantly, the contrast of speckle increases monotonically, and certainly, the increasing rate is much more prominent when contrast dominant NiF is implemented (Fig. 3e). This implies that the focal spot starts to be created at the very first iterations, but it is not completely successful until contrast dominant NiF is applied. These observations at speckle statistics agree well with the object evolution described in Fig. 2 above. In Fig. 3f, we also plotted the intensity value at the focus over optimization iterations. We noted that, although mean intensity of speckle patterns is reduced by contrast dominant NiF, the intensity value at the focal spot is continually increasing during optimizations.

Fig. 3. **Speckle evolution during non-invasive focusing.** Speckle patterns at typical iterations: (a) before optimization, (b) at iteration 320, (c) at iteration 620. Speckle statistics including standard deviation (d) and contrast (e) over iterations. (f) Mean of speckle and intensity at focal spot over iterations. Exposure time of camera is 0.1s.

It is obvious that the speckle mean over optimizing iterations correlates to the excitation intensity at fluorescent object area. As mentioned before, light intensity at sample has been enhanced by 12.12 times after first 320 iterations using mean dominant NiF. Correspondingly, the mean of speckle patterns increases roughly from 500 to 3300 (Fig. 3f blue curve). The role of the subsequent contrast dominant NiF is to shrink the extended focus after mean dominant NiF into a tight focus while the increase of total fluorescent intensity is less emphasized. The actual behavior is even opposite with decreasing fluorescent intensity. If we calculate the light intensity at the object area, intensity is reduced 22.6% at the iteration 620 (Fig. 2f), compared to that at iteration 320 (Fig. 2d), the end of mean dominant NiF. However, while some of the excitation light goes out of object during contrast dominant NiF, some of the excitation light continues to be pulled into the focal spot, increasing its intensity (Fig. 3f red curve). This reduction of intensity at object area, while creating a tighter focal spot, is somewhat related to the essential limit of wavefront shaping, as initially proposed in Ref. [27]. There is a trade-off between focal spot size and intensity enhancement for wavefront shaping based optical focusing through scattering media [27]. For polychromatic light focusing through scattering media (invasive optimization), H.P. Paudel et al theoretically and experimentally investigated the relationship between optimized focus intensity and initial far-field speckle contrast [28]. It is also shown that the intensity enhancement is affected by the bandwidth of feedback signal used in optimization. Our mean dominant NiF is an optimization utilizing narrowband intensity-based feedback, which gives us a high intensity enhancement. The followed contrast dominant NiF reduces the focal spot size, while intensity at focal spot is further increased.

One needs to control the balance between mean (intensity) and contrast (focal spot size) by choosing an appropriate α in optimizations. If standard deviation serves as a cost function in the optimizations, it can be considered as single-step NiF with mean dominance, and our experimental results show a very loose focus [supplementary material Fig. S4], similar to that in Ref. [21]. One can get a tighter focus with larger α coefficient (i.e. more weight on contrast), but then the ultimate intensity at focal point is reduced. One can fine-tune the coefficient α , or even implement an adaptive approach, to adjust this coefficient continuously during

optimization to achieve a better result. It is also advisable to start with mean dominant NiF to bring more excitation laser light into the fluorescent area; thus enhancing the fluorescent signal. There is a high chance that we achieve very little, or even no fluorescent speckle, when we do contrast-dominant NiF first.

We have proposed a non-invasive wavefront shaping method which is able to focus light inside scattering media with the aid of linear fluorescent signal. In the optimization, contrast and mean of fluorescence-generated speckles, are respectively related to focal spot size and intensity enhancement. Optical focus inside scattering media can be non-invasively created as long as contributions of contrast and mean are appropriately controlled in optimizations. Our two-step NiF approach first emphasizes on increasing the excitation intensity at the fluorescent area by mean dominant NiF then reducing the focal spot size by contrast dominant NiF. Micro-scale spot size with hundred-fold intensity enhancement is achieved through strongly scattering media non-invasively. With our proposed technique, the fluorescent speckle pattern from the focusing spot is essentially a PSF. This non-invasively measured PSF allows us to do imaging around the focusing spot by deconvolution [24-25, 29-34], or move the focus spot to nearby positions within the memory effect region as presented in [supplementary material Fig. S2 and S3]. Our non-invasive focusing through scattering media with linear fluorescent feedback could provide a simple and useful tool for various applications in life science such as optical surgery, drug delivery, imaging or light therapy.

See [Supplementary material](#) for supporting content.

We would like to thank the financial support from Singapore Ministry of Education through AcRF Tier1 grant MOE2019-T1-002-087. We also thank Miao Qi and Prof. Wei Lei for lending us the CW laser (Thorlabs TCLDM9) to finish the experiments, and Dr. P. A. SURMAN for careful reading of the manuscript.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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