

## RESEARCH ARTICLE

# AptaFluorescence: An aptamer-based fluorescent imaging protocol for biomolecule visualization

Crystal Chia Yin Ling , Melissa Jane Fullwood \*

School of Biological Sciences, Nanyang Technological University, Singapore, Singapore

\* [mfullwood@ntu.edu.sg](mailto:mfullwood@ntu.edu.sg) OPEN ACCESS

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## Abstract

Immunofluorescence is highly dependent on antibody-antigen interactions for accurate visualization of proteins and other biomolecules within cells. However, obtaining antibodies with high specificity and affinity for their target proteins can be challenging, especially for targets that are complex or naturally present at low levels. Therefore, we developed AptaFluorescence, a protocol that utilizes fluorescently labeled aptamers for *in vitro* biomolecule visualization. Aptamers are single-stranded nucleic acid molecules that fold into three-dimensional structures to bind biomolecules with high specificity. AptaFluorescence targeting the c-MYC protein was evaluated in doxycycline-inducible c-MYC U2OS cells. AptaFluorescence signals were more distinct compared to the diffuse immunofluorescence signals. AptaFluorescence also reliably differentiated doxycycline-treated cells from untreated cells. In conclusion, AptaFluorescence is a novel, easy to perform, aptamer-based protocol that will have broad applicability across various biological endeavours for visualizing biomolecules, especially in cases where antibodies with high affinity and specificity for their target proteins are lacking.

## 1. Introduction

Immunofluorescence (IF) is routinely performed to detect and visualize target proteins within cells by leveraging antibody-antigen interactions [1]. For optimal results, antibodies must possess high specificity and affinity for their targets [2]. However, some proteins of interest, such as c-MYC, have poorly performing antibodies [3] which can limit the consistency of experimental outcomes. Additionally, batch-to-batch variability in antibodies due to their production in immunized animals can further undermine the reproducibility of biological investigations, particularly in experiments involving intrinsically low levels of target proteins [4].

Aptamers are single-stranded nucleic acid molecules that fold into distinct three-dimensional structures for binding to proteins and other biomolecules. Due to their smaller size, aptamers can bind with greater specificity [5] to protein isoforms or epitopes [6, 7] that are often inaccessible to larger antibodies [10]. Aptamers can be designed to target various *in vitro*

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receptors for visualizing subcellular structures [8, 10] and for visualizing protein internalization processes [9]. For monitoring endosomal trafficking, three aptamers were developed to target the transferrin receptor, prostate-specific membrane antigen, and epidermal growth factor receptor, all of which are internalized into endosomes after binding their respective ligands [10]. Compared to antibodies, the images obtained with the aptamers offered greater clarity in structural features such as circular endosomal shapes and thin endosomal tubules [10]. Considering the enhanced imaging capability of aptamers, we hypothesized that aptamers could be leveraged in an easy-to-perform protocol, analogous to traditional immunofluorescence, for effective protein visualization.

Here, we introduce AptaFluorescence, an aptamer-based fluorescence protocol developed for *in vitro* protein visualization. We tested a fluorescently labeled aptamer for visualizing c-MYC in doxycycline-inducible c-MYC U2OS cells, where doxycycline treatment was previously confirmed to increase c-MYC levels in these cells [14]. Compared to conventional immunofluorescence using an anti-c-MYC antibody, AptaFluorescence yielded higher resolution images with more defined c-MYC signals. AptaFluorescence was also sensitive to variations in endogenous c-MYC levels, as indicated by distinct fluorescence intensities differentiating doxycycline-treated cells from untreated cells. Hence, AptaFluorescence is a promising and easy-to-perform aptamer-based protocol that can enable high-resolution imaging across diverse molecular targets.

## 2. Materials and methods

### 2.1. Materials

For cell culture, Dulbecco's modified Eagle's medium (DMEM) and tetracycline-free fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific. Penicillin-streptomycin solution was purchased from GE Hyclone. Phosphate-buffered saline (PBS) and trypsin were purchased from Gibco.

For cell imaging, 8 well chamber glass slides were sourced from ThermoFisher. 4% paraformaldehyde in PBS, tris-buffered saline (TBS), and ProLong<sup>TM</sup> Glass Antifade Mountant were purchased from Thermo Fisher Scientific. Tween-20 was purchased from Bio Basic. Bovine serum albumin (BSA) and 4',6-diamino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich.

For immunofluorescence, primary antibody against c-MYC (ab32072) and IgG (ab37355) were purchased from Abcam. The secondary goat anti-mouse IgG antibody, which was conjugated to a green fluorophore (ab150113) was also purchased from Abcam.

For AptaFluorescence, the sequences of the targeted and non-targeted (NT) aptamers were sourced from the literature [11]. The aptamer sequence previously validated for its specificity and affinity towards c-MYC was selected as the targeted aptamer in this study [11]. In contrast, the aptamer comprising random sequences was selected as the control NT aptamer due to its demonstrated lack of specificity for c-MYC [11]. Both the targeted and NT aptamers were modified with a red 5' Cy3<sup>TM</sup> fluorescent dye and synthesized as lyophilized powders by Integrated DNA Technologies. The sequences of the aptamers are provided in [S1 Table](#) of the Supplementary Material.

### 2.2. Cell culture

The human osteosarcoma U2OS cell line with a doxycycline-inducible MYC system was received as a gift from the lab of Elmar Wolf, Universität Würzburg, Germany. U2OS cells were cultured in DMEM supplemented with 10% tetracycline-free FBS and 1% penicillin-streptomycin solution at 37°C in a humidified incubator containing 5% CO<sub>2</sub>.

### 2.3 Immunofluorescence

U2OS cells were seeded at a density of 10,000 to 20,000 cells per well into an 8-well chamber glass slide and incubated overnight. Some cells were treated with doxycycline, which was added to a final concentration of 1  $\mu\text{g}/\text{mL}$  to elevate endogenous c-MYC levels. The remaining untreated cells received media as a control. Following an overnight incubation, the cells were fixed with 4% paraformaldehyde for 30 minutes and washed with PBS. Then, the cells were incubated for 1 hour in blocking buffer (5% BSA in Tris Buffered Saline with 0.2% Tween-20 (TBST)) to block non-specific protein binding sites.

Both the primary anti-c-MYC antibody and non-specific IgG antibody were diluted by 100 times in blocking buffer (5% BSA in 0.2% TBST) and introduced into the cells. Following an overnight incubation at 4°C, the cells were washed with TBST thrice. The secondary goat anti-mouse IgG antibody was then incubated with the cells at room temperature for 1 hour, followed by three washes with TBST. The nuclei were then stained with DAPI for 10 minutes, then washed twice with TBST. The silicone sealing gasket was removed, and the mounting media and coverslips were applied. The glass slide was cured at 4°C overnight before imaging with a Zeiss live cell observer at 20x, 40x and 63x magnification levels.

### 2.4 AptaFluorescence

U2OS cells were seeded at a density of 10,000 to 20,000 cells per well into an 8-well chamber glass slide and incubated overnight. Some cells were treated with doxycycline, which was added to a final concentration of 1  $\mu\text{g}/\text{mL}$  to elevate endogenous c-MYC levels. The remaining untreated cells received media as a control. Following an overnight incubation, the cells were fixed with 4% paraformaldehyde for 30 minutes and washed with PBS. The cells were incubated in blocking buffer (5% BSA in TBST) for 1 hour to block non-specific protein binding sites.

Both the c-MYC and NT aptamers, synthesized as lyophilized powders, were reconstituted to 0.188 mg/mL with nuclease-free water to match the stock concentration of the anti-c-MYC antibody. Then, the aptamers were further diluted by 50 times in blocking buffer (5% BSA in 0.2% TBST) and introduced into the cells. Following an overnight incubation at 4°C, the cells were washed with TBST thrice to remove unbound aptamers. The nuclei were then stained with DAPI for 10 minutes, followed by two washes with TBST. Then, the silicone sealing gasket was removed, and the mounting media and coverslips were applied. The glass slide was cured at 4°C overnight before imaging with a Zeiss live cell observer at 20x, 40x and 63x magnification levels.

### 2.5 Quantification of fluorescence signals with ImageJ

The ImageJ software was used for image processing and quantification of fluorescence signals. Each image was separated into individual color channels to analyse fluorescence signals specific to each fluorescent label. Pixel intensities in the green and red channels were measured using the mean gray value function for the immunofluorescence and AptaFluorescence images respectively.

Fluorescence signals were quantified within manually delineated cellular boundaries using the freeform selection tool in ImageJ. Cells that were overlapping or only partially visible in the image were excluded from the analysis. Background fluorescence was measured from areas immediately adjacent to the delineated cellular boundaries. For each cell, the corrected total cellular fluorescence (CTCF) [12] was calculated using the following Eq (1):

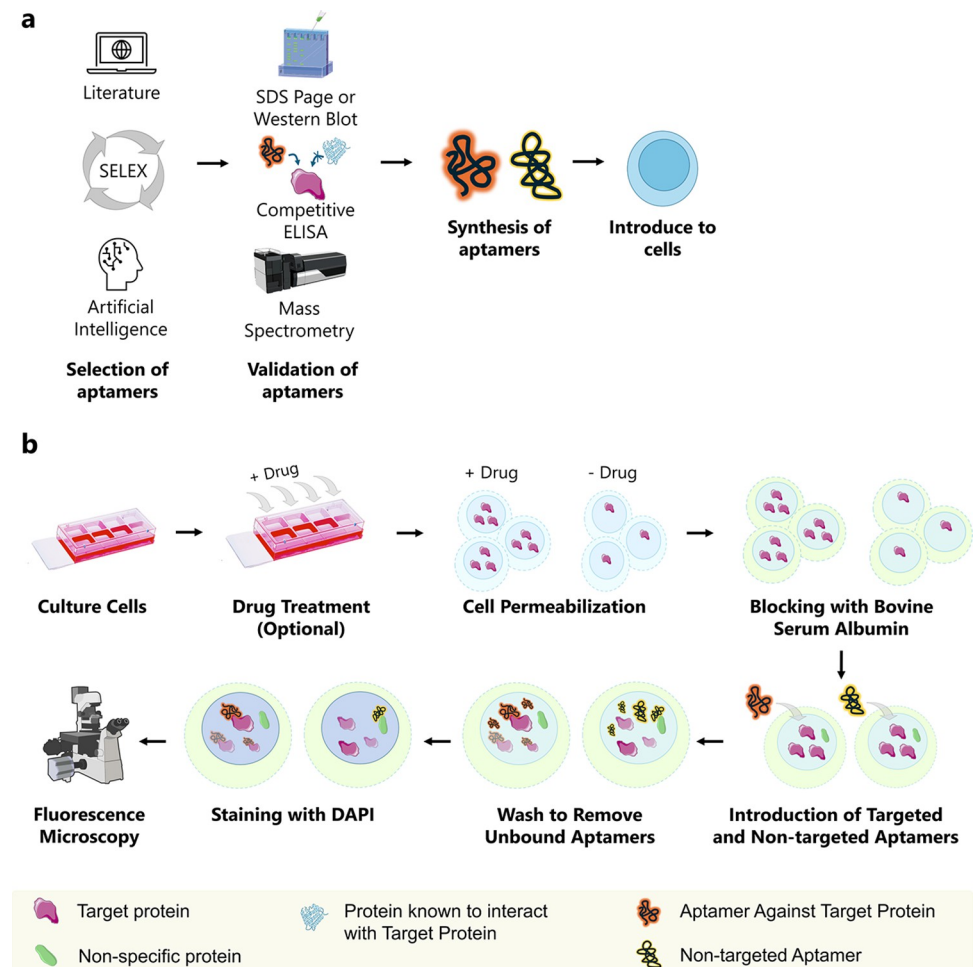
$$\text{CTCF} = \text{Integrated Density} - (\text{Area of cell} \times \text{Mean background fluorescence}) \quad (1)$$

For each image, CTCF values were summed across all cells and normalized to the total number of cells analyzed. Three images were analyzed for each experimental condition. The normalized CTCF values were then averaged across the technical triplicates and reported as mean fluorescence intensity  $\pm$  standard deviation. Significant differences between doxycycline-treated and untreated cells were assessed using Student's t-test.

### 3. Results

#### 3.1. AptaFluorescence reproduces immunofluorescence signals with higher resolution

The AptaFluorescence protocol comprises two main parts: (i) selection, validation, and synthesis of aptamers (Fig 1A) and (ii) preparation of cells for fluorescence microscopy (Fig 1B, S2 Fig). A targeted aptamer exhibiting specificity to the target protein is first identified from literature, Systematic Evolution of Ligands by EXponential enrichment (SELEX), or artificial intelligence, followed by validation using Competitive ELISA and Mass Spectrometry, and then synthesized and introduced to cells.



**Fig 1. Overview of the AptaFluorescence protocol.** (a) Selection, Validation, and Synthesis of Aptamers. (b) Preparation of Cells for Fluorescence Microscopy. Aptamer sequences with specificity to target proteins are identified, validated for their specificity, and synthesized. Cells are cultured and may be treated with drugs to elevate endogenous protein levels. The cells are then permeabilized, and non-specific protein binding sites are blocked before the fluorescently labeled aptamers are introduced. Unbound aptamers are removed by washing, and the nuclei are stained with DAPI for fluorescence imaging.

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intelligence models (Fig 1A). Additionally, a non-targeted control aptamer, typically comprising of random nucleotide sequences, is also selected that exhibits minimal specificity to the target protein (Fig 1A). The specificity of the aptamers for the target protein is validated through assays such as SDS-PAGE and liquid-chromatography mass-spectrometry before they are synthesized with fluorescent dyes (Fig 1A). In AptaFluorescence, cells are prepared similarly to immunofluorescence where they are permeabilized and non-specific protein binding sites are blocked (Fig 1B, S1 Fig). However, fluorescently labeled aptamers are introduced into cells instead of antibodies (Fig 1B, S1 Fig). Thereafter, the excess aptamers are removed by washing, and the slide is prepared for fluorescence microscopy. A more detailed AptaFluorescence protocol is described in the Supplementary Material (S2 Fig).

The AptaFluorescence protocol was applied to detect c-MYC in doxycycline-inducible c-MYC U2OS human osteosarcoma cells. c-MYC, a transcription factor aberrantly expressed in over 70% of human cancers [13], was engineered into a doxycycline-inducible cassette in these cells, leading to increased c-MYC expression upon doxycycline treatment [14]. As immunofluorescence is the current gold standard for *in vitro* biomolecule visualization, it was also performed with anti-c-MYC and IgG antibodies serving as positive and negative controls respectively.

AptaFluorescence using c-MYC aptamers appeared to capture similar fluorescence signals as the c-MYC antibody at all magnifications tested (Fig 2). AptaFluorescence signals were more distinct and revealed greater subcellular localization information compared to the diffuse immunofluorescence signals (Fig 2).

### 3.2 AptaFluorescence is specific and sensitive to endogenous target protein levels

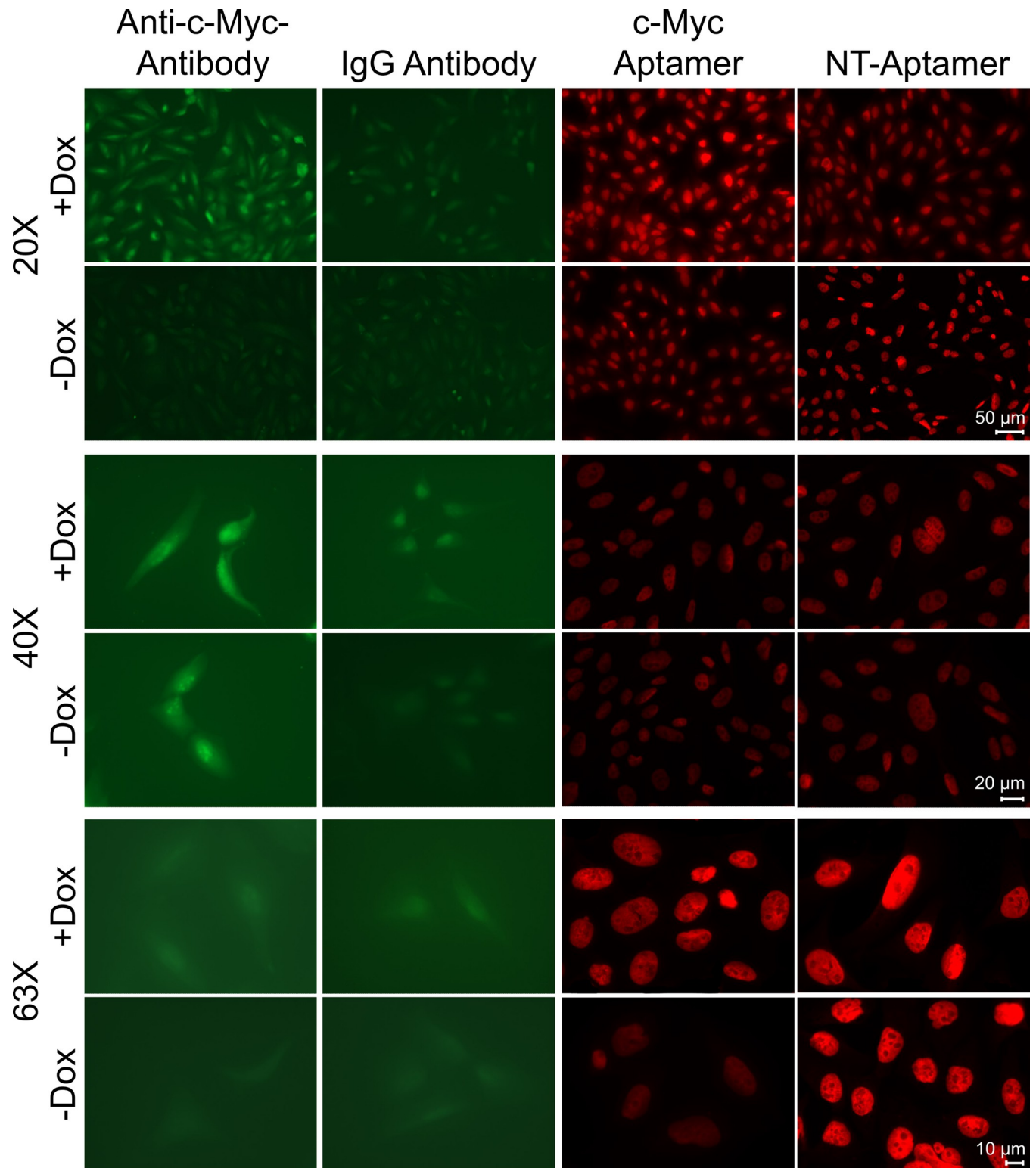
To assess whether AptaFluorescence can reliably distinguish between varying endogenous protein levels, U2OS cells were treated with doxycycline to increase c-MYC expression for comparison with untreated U2OS cells. Mean fluorescence intensity for each experimental condition was determined from three technical replicates at 63x magnification (Figs 3 and 4), with the results summarized in Table 1.

Significant differences in mean fluorescence intensity at the 5% significance level ( $p < 0.05$ ; Table 1) between doxycycline-treated and untreated cells at 63x magnification for both the c-MYC aptamer (Fig 3C) and the anti-c-MYC antibody (Fig 4C) were observed. In contrast, no significant differences ( $p > 0.05$ ; Table 1) in mean fluorescence intensity between doxycycline-treated and untreated cells for both the NT aptamer (Fig 3C) and IgG antibody (Fig 4C) were observed.

## 4. Discussion

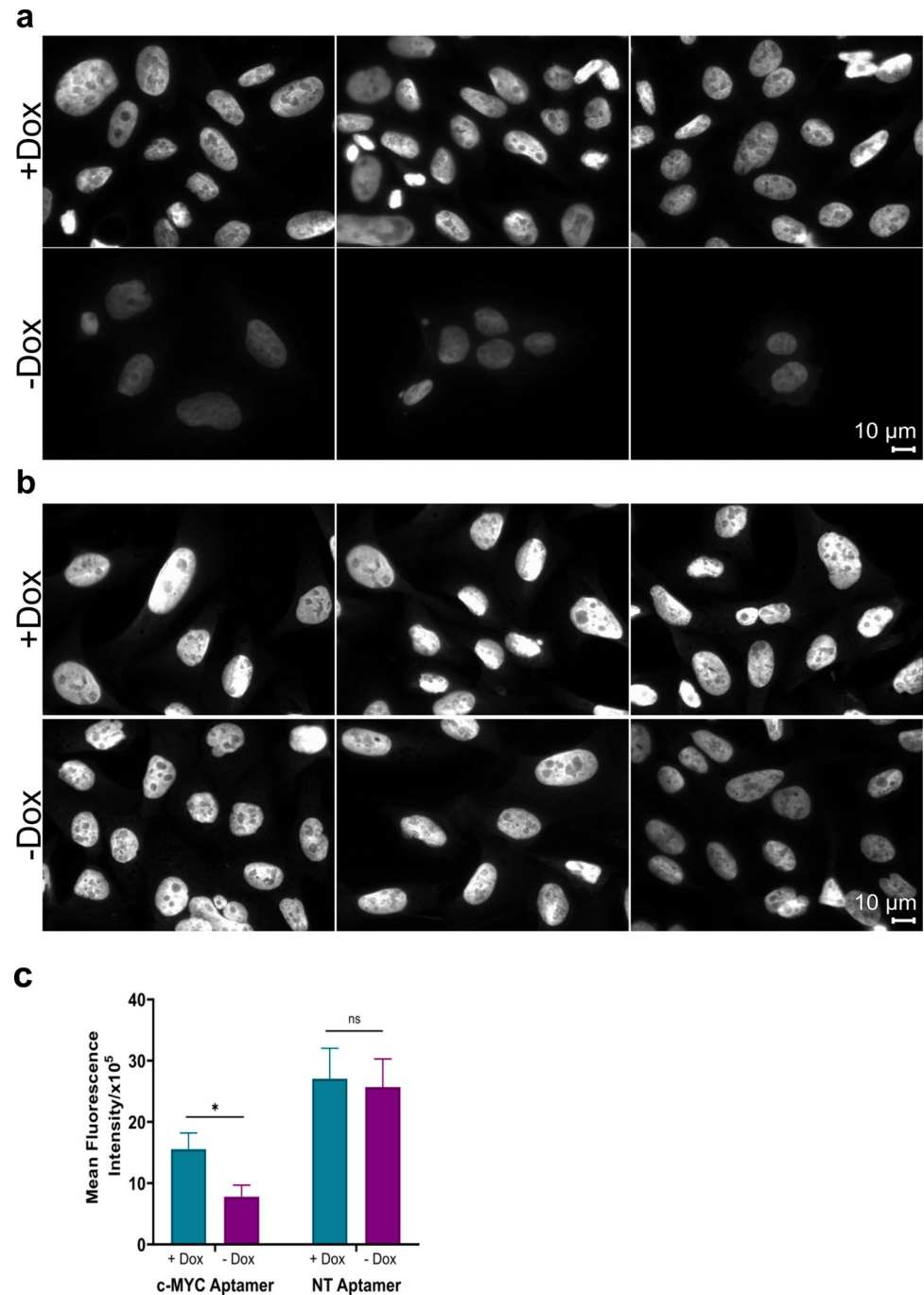
AptaFluorescence, an aptamer-based immunofluorescence protocol, was developed as an alternative to antibody-based methods for visualizing biomolecules. In conventional immunofluorescence, primary antibodies are introduced into cells to bind to the target protein. After an incubation period, fluorescently labeled secondary antibodies are introduced to visualize the target protein. This method relies on the binding affinity between antibodies and specific proteins but is constrained by the availability of suitable antibodies. By utilizing fluorescently labeled aptamers, AptaFluorescence overcomes this limitation and offers a viable alternative to antibody-based methods.

We applied AptaFluorescence to detect c-MYC in doxycycline-inducible U2OS cells. The c-MYC aptamer reproduced the fluorescence signals observed with the anti-c-MYC antibody (Fig 2), but with higher imaging resolution than traditional immunofluorescence techniques



**Fig 2. Representative microscopy images of doxycycline-treated (+Dox) and untreated (-Dox) U2OS cells incubated with anti-c-MYC antibody, IgG antibody, c-MYC aptamer, and non-targeted aptamer at 20x, 40x, and 63x magnification levels. Green fluorescence signals correspond to AlexaFluor™ 488 dyes conjugated to secondary goat anti-mouse IgG antibodies. Red fluorescence signals correspond to Cy3 dyes conjugated to c-MYC and NT aptamers.**

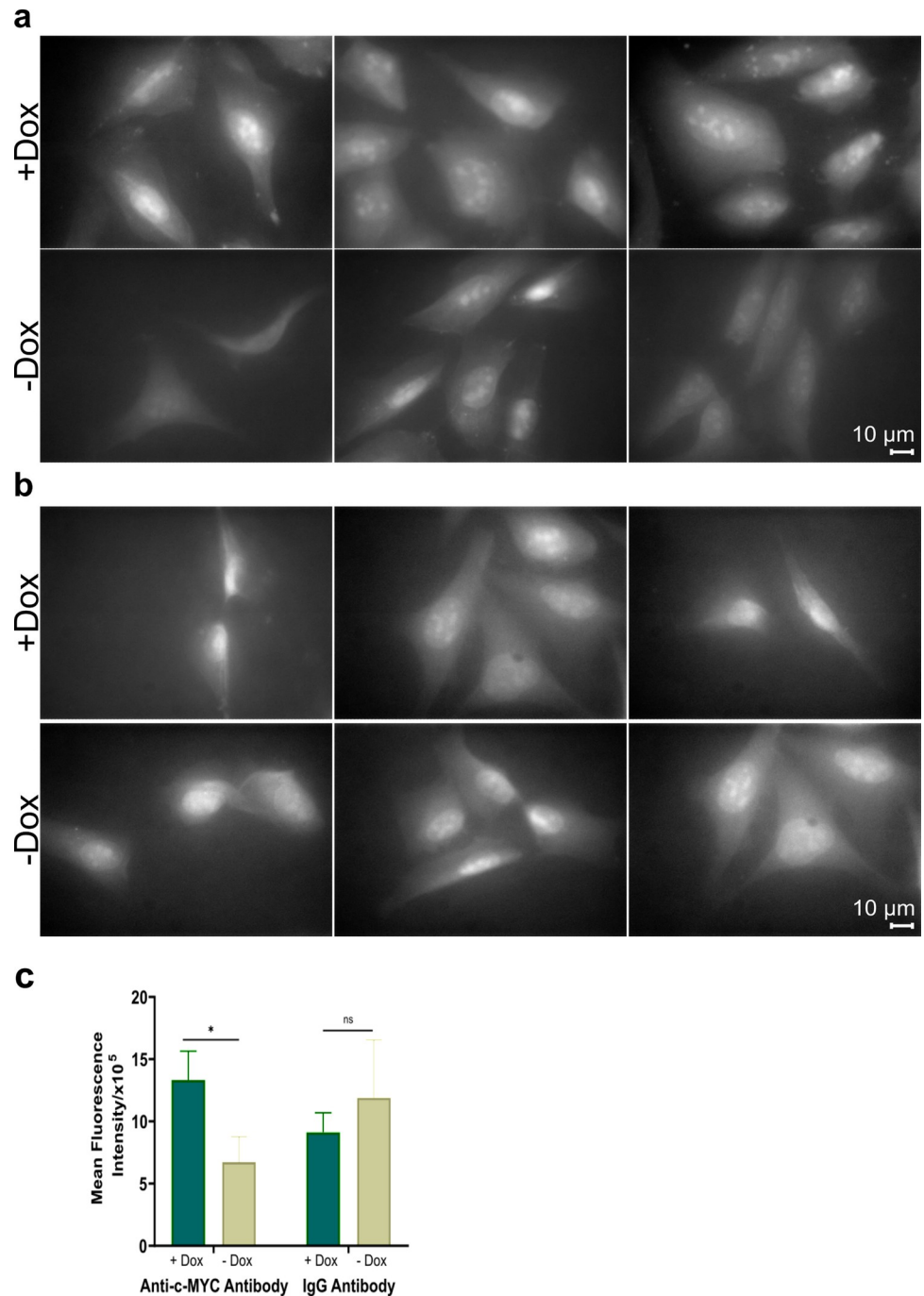
<https://doi.org/10.1371/journal.pone.0316359.g002>



**Fig 3.** AptaFluorescence images captured at 63x magnification for (a) c-MYC aptamer and (b) NT aptamer. (c) Bar chart of mean fluorescence intensity for each condition, with error bars representing standard deviation. The images of the replicates are shown sequentially from left to right within each row. Statistical significance was assessed using Student's t-test (\* $p < 0.05$ , ns: not significant).

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(Fig 2). The distinct signals produced by the c-MYC aptamer can be attributed to its higher specificity for the c-MYC protein compared to the larger antibody (Fig 2). This finding aligns with the observations of Opazo et al [10], who suggested that the smaller size of aptamers increases their accessibility to target proteins, thereby revealing greater structural information



**Fig 4.** Immunofluorescence images captured at 63x magnification for (a) anti c-MYC and (b) IgG antibodies. (c) Bar chart of mean fluorescence intensity for each condition, with error bars representing standard deviation. The images of the replicates are shown sequentially from left to right within each row. Statistical significance was assessed using Student's t-test (\* $p < 0.05$ , ns: not significant).

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in super-resolution images [10]. Notably, the fluorescence signals from the anti-c-Myc antibody appeared diffuse at all magnifications tested (Fig 2) due to weak antibody-antigen binding affinities, which typically lead to non-specific binding and increased background noise [3, 15]. Compared to traditional antibodies, the smaller size and higher specificity of aptamers

**Table 1. Mean fluorescence intensities ( $\times 10^5$ ) of doxycycline-treated (+ Dox, n = 3) and untreated (- Dox, n = 3) U2OS cells incubated with anti-c-MYC antibody, IgG antibody, c-MYC aptamer and non-targeted aptamer at 63x magnification.**

	Mean Fluorescence Intensity ( $\times 10^5$ )		p-value
	+ Dox (n = 3)	- Dox (n = 3)	
Anti-c-MYC antibody	13.33 $\pm$ 2.31	6.74 $\pm$ 2.04	<b>0.02</b>
IgG antibody	9.14 $\pm$ 1.57	11.92 $\pm$ 4.63	0.40
c-MYC aptamer	15.58 $\pm$ 2.64	7.80 $\pm$ 1.87	<b>0.01</b>
Non-targeted aptamer	27.08 $\pm$ 4.96	25.69 $\pm$ 4.58	0.74

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provide distinct advantages in producing sharper and more precise fluorescence signals. This establishes AptaFluorescence as an effective tool for *in vitro* visualization of target proteins.

Additionally, AptaFluorescence exhibited sensitivity to endogenous c-MYC levels, as evidenced by a significant difference in mean fluorescence intensity at the 5% significance level ( $p < 0.05$ ) between doxycycline-treated and untreated cells with the c-MYC aptamer (Table 1, Fig 3A and 3C). Both the anti-c-MYC antibody and the c-MYC aptamer effectively distinguished varying levels of c-MYC expression, with elevated c-MYC levels following doxycycline treatment correlating with higher mean fluorescence intensities (Table 1, Fig 3A and 3A). In contrast, the mean fluorescence intensities for both the IgG antibody and NT aptamer showed no significant difference ( $p > 0.05$ ) between doxycycline-treated and untreated cells (Table 1, Figs 3B and 4B). Since the NT aptamer and the IgG antibody are insensitive to changes in c-MYC levels, this indicates their lack of specificity to c-MYC. Overall, these results lend further support to fluorescently labeled aptamers as a viable alternative to antibodies for biomolecule imaging.

Despite the extensive use of aptamers in fluorescence imaging for applications such as organelle imaging [8], monitoring endosomal trafficking [10], real-time visualization of protein internalization [9], and immunohistology [16], their use as an alternative to antibodies in immunofluorescence experiments remains unexplored. A key limitation of immunofluorescence is the suboptimal affinity or specificity of antibodies for their protein targets, which can result in diffuse signals and false positives, particularly when target proteins are expressed at low levels [15]. In such cases, AptaFluorescence, which utilizes fluorescently labeled aptamers, can offer unprecedented structural resolution and stands as a promising alternative to traditional immunofluorescence.

Beyond enhancing imaging resolution, aptamers also address additional challenges associated with antibody production. Antibodies are conventionally extracted and purified from the serum of immunized animals. This process has ethical implications, introduces significant variability, and requires long production time. In contrast, aptamers can be produced rapidly through *in vitro* systems that are animal-free, cost-effective and scalable. Furthermore, their ease of chemical modification [17] enhances their adaptability for a wide range of advanced applications and investigations. Considering these advantages, we anticipate greater adoption of aptamers for *in vitro* fluorescence microscopy approaches, especially in instances where highly specific antibodies are unavailable or impractical to use.

Given the demonstrated advantages of AptaFluorescence, aptamers and antibodies are likely to become complementary tools for *in vitro* imaging in the future. Through AptaFluorescence, researchers can leverage fluorescently labeled aptamers to enhance the quality and reliability of biological investigations. We hope that AptaFluorescence will facilitate more cost-effective and rapid *in vitro* biomolecule visualization for advancing future biological and clinical research.

## Supporting information

**S1 Fig.** Schematic of (a) Immunofluorescence and (b) AptaFluorescence protocols. AptaFluorescence is an alternative aptamer-based biomolecule visualization protocol that introduces fluorescent aptamers instead of antibodies for in vitro biomolecule visualization. (TIF)

**S2 Fig. Overview of the AptaFluorescence protocol. (a) Selection, validation and synthesis of aptamers.** An aptamer sequence that binds to the target protein with high specificity and affinity is identified. A non-targeted aptamer comprising random sequences with little to no affinity to the target protein is also identified. Using various assays, the aptamers are validated for their specificity to target proteins. Thereafter, both the targeted and non-targeted aptamers are synthesized with fluorescent labels. **(b) Preparation of cells for fluorescence imaging.** Cells are cultured and may be treated with drugs to elevate endogenous target protein levels. The cells are permeabilized, and non-specific protein binding sites are blocked. Both the targeted and non-targeted aptamers are introduced into the cells. The unbound aptamers are removed by washing, and the nuclei are stained with DAPI for fluorescence microscopy. (TIF)

**S1 Table. Sequences of targeted and non-targeted aptamers.** (DOCX)

## Author Contributions

**Conceptualization:** Melissa Jane Fullwood.

**Formal analysis:** Crystal Chia Yin Ling, Melissa Jane Fullwood.

**Investigation:** Crystal Chia Yin Ling.

**Methodology:** Crystal Chia Yin Ling.

**Resources:** Melissa Jane Fullwood.

**Supervision:** Melissa Jane Fullwood.

**Writing – original draft:** Crystal Chia Yin Ling, Melissa Jane Fullwood.

**Writing – review & editing:** Crystal Chia Yin Ling, Melissa Jane Fullwood.

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