Signal enhancement in immunofluorescence via polydopamine deposition

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Abstract

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Current achievements in biomedical science rely on the development and improvement of contemporary technologies. Therefore, in the context of biomedical detection, there's a growing need for researching technologies with escalated sensitivity and precision, high speed, low cost, and multiplexing capacity. For instance, immunofluorescence has been a powerful tool providing valuable, high dimensional information from biomarkers of interest in patients' biopsy for decades. However, traditional immunofluorescence labeling lacks adjustability for signal amplification, thus its application has been largely limited in visualizing targets with sufficient abundance above detection limit of the assay. In this report, we described the development of a polydopamine-based immunofluorescence technology, which localize targets of interest in a rapid and multiplexed manner, along with amplification potency for target with low abundance. In summary, the work demonstrated in this report provides a possible solution for rare target multiplexed detection and a strong evidence for its potential to be adapted into clinical applications.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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INTRODUCTION

Immunohistochemistry is a powerful technique widely used in both fundamental research and clinical diagnosis to localized the distribution of biomarkers and visualize histological structure in biomedical samples. The foundation of IHC is to exploit the specificity of antibodies to target of interest (antigen) and to achieve visualization by introducing reporter molecules. There are two strategies to introduce reporter: conjugating reporter directly onto secondary antibody (Figure. 1a) or introducing reporter through other molecular interactions or enzymatic reaction (Figure.1b). The advantage of unamplified labeling strategy includes short assay time, simple procedure and less background signal caused by non-specific staining. However, since the labeling efficiency of this method is highly relying on the reporter loading capacity of the secondary antibody, this strategy meets difficulty when visualizing targets with low abundance. Another strategy is amplified labeling methods that could overcome this problem. In this case, an enzyme is conjugate onto secondary antibody, which could react with chromogenic substrate to develop various types of signals in the proximity of target. This strategy provides adaptability for signal enhancement and applicability for clinical use.

The most important mission in IHC is to increase the sensitivity and reproducibility of the current technologies[1,2]. Major focus lied in either amplifying true signal generated by targets [3] or eliminating background signal from various sources, for instance, autofluorescence from biological sample, non-specific staining and instrumental bias. Efforts have been made in developing tools to amplify the true signal for target of interest. [4,5] Multi-layer antibody method was first introduced in 1988 using multiple cycles of primary antibody-labeled secondary antibody to maximize the number of target-associated antibodies. [6] A polymer-based

amplification method using dextran backbone to recruit HRP was also developed and applied in immunohistochemistry [7,8]. Avidin-biotin systems were also exploited to increase the number of reporters originated from target antigen. [9–11] A catalyzed reporter deposition method was developed in 1989 using HRP-labeled secondary antibody to catalyze the deposition of biotin-labeled phenol. This biotin-labeled phenol molecules could then react to streptavidin-labeled HRP, resulting in an enhanced signal induced by the same amount of target. [12] Similarly, in 1996, another method exploiting biotinylated-tyramide and fluorophore-labeled tyramide was reported with capacity to improve staining efficiency by 10- to 100- fold [4]. However, limitations still existed in those methods described above, for instance, high background signal caused by secondary antibody non-specific binding, background signal generated by endogenous biotin or endogenous peroxidase, lack of adaptability for multiplexed platform.

This report described a rapid sensitive amplification technology that could be applied in immunofluorescence detection. This technology is based on the polymerization reaction of polydopamine (PDA), which is a versatile surface coating material highly adaptable to a wide range of materials[13]. Previous work in our lab discovered that the polymerization rate of PDA could be increased by over 300-fold in presence of HRP. After exposed to HRP in an aqueous condition with hydrogen peroxide, dopamine molecules undergo rapid polymerization and form PDA deposit in the proximity of HRP. Through amine-reactive sites exposed on PDA, more HRP molecules could be recruited and absorbed onto PDA complex, resulting in amplified enzymatic signal level at the end of the detection. [14](Figure.2a) However, the amplification efficiency of this method is largely limited by the recognition step between amine-functionalized molecule and amine-reactive sites on PDA[15,16], which is a reaction sensitive to environmental condition and need long time to guarantee sufficient amount of linking, which is disadvantageous

in clinical applications**.** To improve the performance of this technology, here we proposed two alternative strategies. One is to utilized biotin-streptavidin interaction between biotinylated-dopamine and streptavidin-labeled fluorophores (Figure.2b). In this case, biotin-labeled dopamine is polymerized in the proximity of HRP and the reporters labeled with streptavidin are introduced through the recognition between biotin-streptavidin. (Figure.2c). Another strategy is to covalently link dopamine with fluorophore, which could result in site-specific deposition of fluorophore directly after the polymerization of PDA. Both strategies utilized NHS ester conjugation chemistry linking NHS- functionalized molecules (NHS-biotin or NHS-fluorophore) with the amine-contained dopamine monomer (Figure.3) to generate the reporter molecules: biotin-DA and fluorophore-DA.

Additionally, multiplexing potential has been observed with fluorophore-DA system. Multiplexed single molecule immunofluorescence has been recognized as a promising tool to provide valuable proteomics information at single-cell level by profiling numerous protein targets within the original cellular context. This allows the visualization of not only single molecule of interest but also the molecular interaction revealed under single-molecule resolution, which is a powerful tool in the study of dynamic receptor interactions [17], interaction of signaling molecules[18], and transcription factor interaction with DNA[18,19]. Combined with multispectral imaging platform, frequently used fluorophores, for instance, Alexa Fluor series and Bella Fluor series, could be separated into different channels based on distinctive spectral profile and be applied in labeling multiple targets without disturbance in a multiplexed staining setting. (Figure.4)

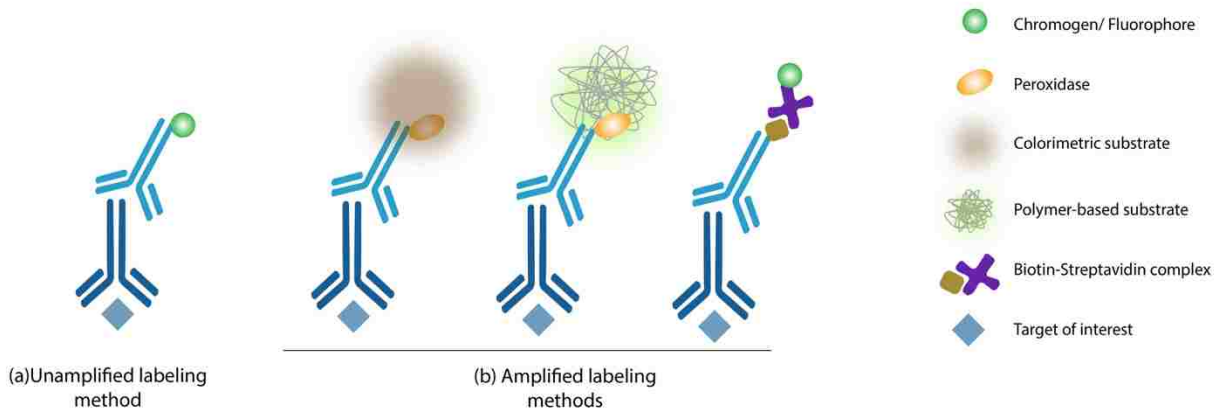

Figure 1 Schematic of unamplified labeling vs amplified labeling strategies

(a) Unamplified labeling with 1st antibody (dark blue) targeting target of interest, 2nd antibody (light blue) directly conjugated with chromogenic/ fluorescent reporter, binding to antigenic sites on 1st antibody. (b) In contrast, other strategies, from left to right, enzymatic chromogenic process, polymer-based color development or biotin-streptavidin complex amplification was also used in an amplified manner.

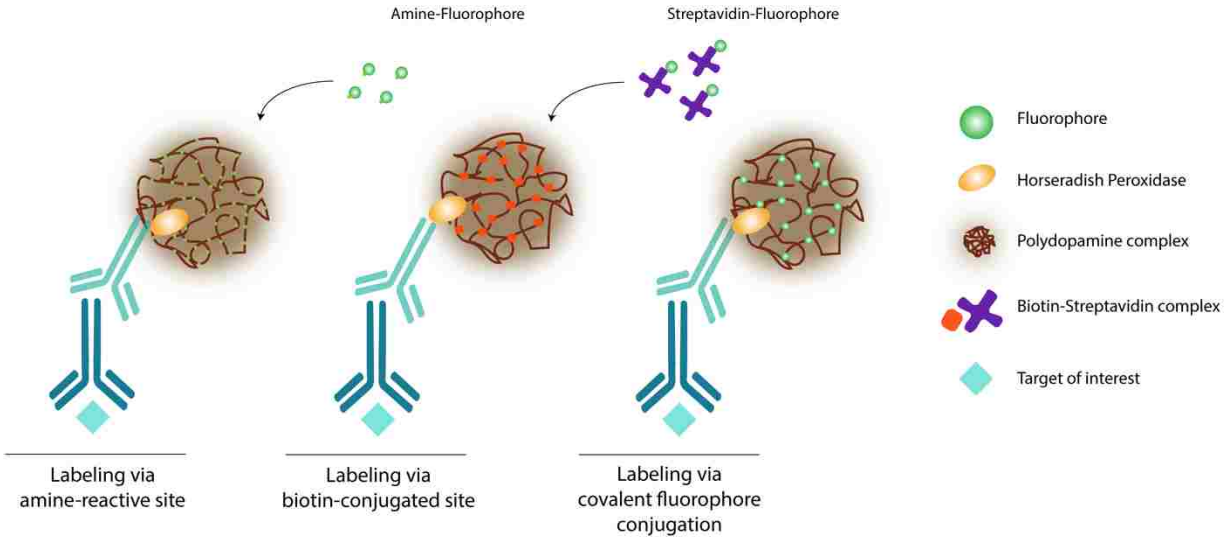

Figure 2 Schematic of polydopamine-based amplification strategies

(a)The strategy described by Junwei Li in his previous work using amine-reactive sites (green dots) on PDA to recruit amine-functionalized fluorophore. (b) Amplification method using biotin-labeled sites (orange squares) on PDA to absorb streptavidin-labeled fluorophore. (c) Amplification method using fluorophore-linked PDA to generate fluorescence directly after polymerization.

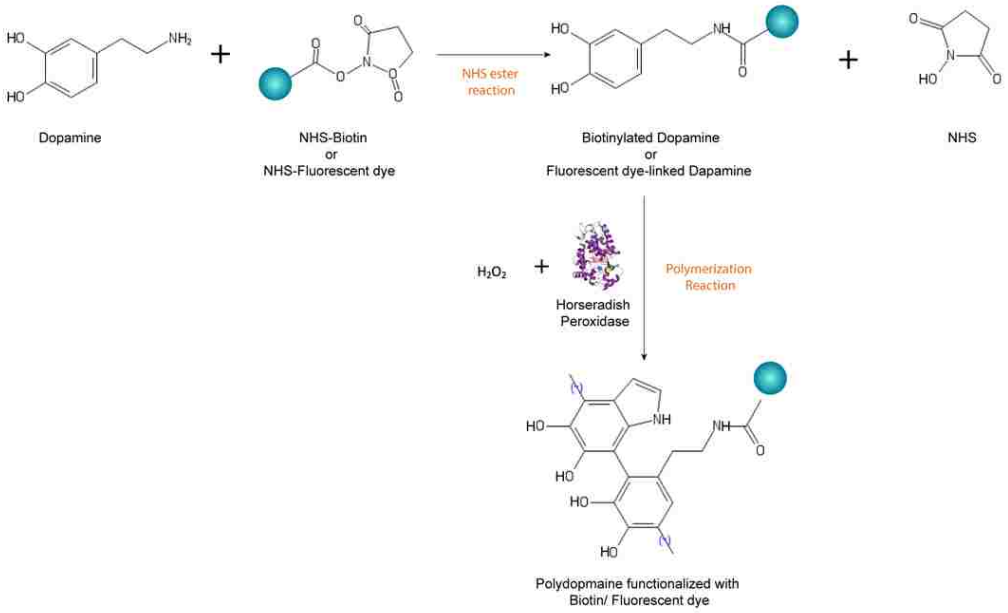

Figure 3 Schematic of conjugation chemistry for DA-biotin and DA-fluorophore complex
The NHS ester reaction is applied in this conjugation. Dopamine functions as amine-group provider that reacts with either NHS-labeled biotin or NHS-labeled fluorophore in alkaline condition. The reaction will produce biotin- or fluorophore-dopamine complex linked with stable amide bond, releasing NHS into reaction solution. The product of conjugation is then polymerized into PDA complex in the presence of HRP.

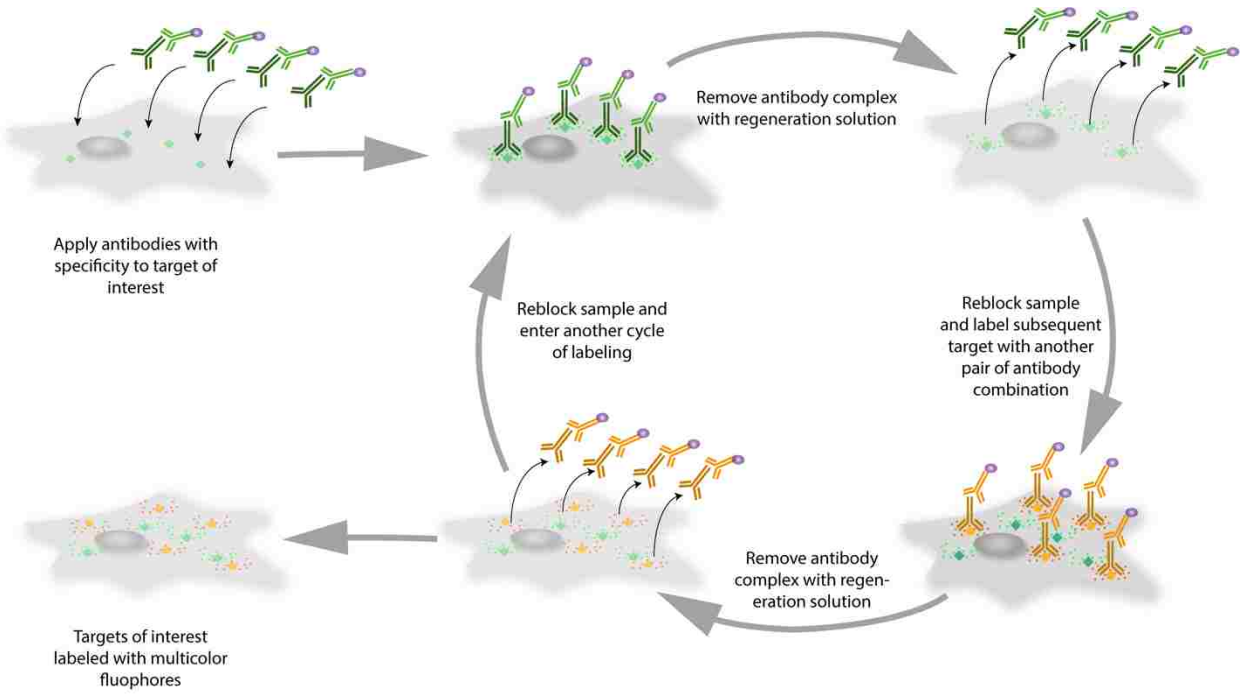

Figure 4 Schematic of DA-fluorophore-based multicolor staining method

Multicolor staining starts with applying antibodies (green series) with specificity to target of interests (green square). After developing fluorescence with DA-fluorophore, antibodies are removed by regeneration buffer while DA-fluorophore deposit stays in vicinity of target of interest. Reblock the sample and apply another round of antibodies to localize another target. Repeat regeneration procedure and maintain the fluorescence in the sample. After several cycles of repetitive staining, sample is labeled with multiple fluorophores and ready for imaging.

ABBREVIATIONS

IHC**,** immunohistochemistry; DA, dopamine; PDA, polydopamine; NHS, N-Hydroxysuccinimide; DMF, dimethylformamide; TEA, triethylamine; BF, Bella Fluor, AF; Alexa Fluor; FBS, fetal bovine serum; DTAC, Dodecyltrimethylammonium chloride; BSA, bovine serum albumin; TBS, Tris-buffered saline, TBST, Tris-buffered saline with Triton-X; HRP, horseradish peroxidase; FFPE, formalin-fixed paraffin-embedded; DAB, 3,3' Diaminobenzidine.

MATERIALS

Dopamine hydrochloride, NHS-biotin (Thermo, 20217), Anhydrous DMF (Acros, 326870010), TEA (Thermo, 25108), BF594-NHS (Setareh biotech, 7799), BF488-NHS(Setareh biotech, 8050), AF680-NHS (Thermo, A37567), 5(6) TAMRA-NHS (Setareh Biotech, 6267), paraformaldehyde, Blockaid Blocking Solution (Thermo, B10710), Image-iT™ FX Signal Enhancer(Thermo, I36933), Trilogy™ (Cell Marque, 920P), DAB (Thermo, 34002), Rabbit anti-LaminA antibody (Sigma, 1293), Rabbit anti-cox4 antibody (Santa Cruz, 292052), Rabbit anti-Histone H3 antibody (Santa Cruz, 8654), Rabbit anti-HSP90 antibody (Santa Cruz, 7947), Rabbit anti-Nup98 antibody (Santa Cruz, 30112), Rabbit anti-GAPDH antibody (Santa Cruz, 25778), Goat anti-rabbit IgG (H+L) conjugated with HRP (Invitrogen, 656120), AF555-streptavidin (Life technology, S21381), FITC-streptavidin (eBioscience, 11431787), Goat anti-rabbit (H+L) with QD655 (Life technology, Q11421), Goat anti-rabbit (H+L) with AF555 (Life technology, A12429), Goat anti-rabbit alpha-tubulin (Cell signaling, 21253), Goat anti-rabbit beta-tubulin (Cell signaling, 21285), Goat anti-mouse IgG with poly HRP (Thermo, 32230)

METHODS

PREPARATION OF DOPAMINE-BIOTIN CONJUGATION

Dissolve 4.5mg dopamine hydrochloride with 0.225 mL anhydrous DMF and vortex the tube to mix it homogeneously. Dissolve 2mg NHS-biotin with 0.2mL anhydrous DMF in another tube and vortex the tube. Transfer the dopamine reaction solution to NHS-biotin solution and add 4uL TEA into the mixed solution. Vortex the tube to mix the solution. Cover the tube with aluminum film and react at RT overnight with continuous stirring. Store immediately at -20C after the synthesis for future use.

The final concentration of dopamine in the reaction solution is 20mg/mL, equivalent to 56.78mM and the final concentration of NHS-biotin in system is 10mg/mL, equivalent to 14.5 mM. (Table.1)

PREPARATION OF DOPAMINE-DYE CONJUGATION

Dissolve 0.9mg dopamine hydrochloride with 0.1mL anhydrous DMF and vortex the tube to mix it homogeneously. Dissolve 1mg NHS-BF594 with 0.1mL anhydrous DMF in another centrifuge tube and vortex the tube. Transfer the dopamine reaction solution to NHS-AF594 solution and add 5uL TEA into mixed solution. Vortex the tube to mix the solution. Cover the tube with aluminum film and react at RT overnight with continuous stirring. Store immediately at -20C after the synthesis for future use.

The final concentration of dopamine in the reaction is 9mg/mL, equivalent to 48mM and the final concentration of NHS-AF594 is 10mg/mL, equivalent to 12mM. For the conjugation of DA-BF488, DA-AF680 and DA-TAMRA, the procedure is the same as previous described. All

the conjugation follow same final concentration of dopamine (48mM) and keep the molarity ratio of dopamine to dye to be 4:1.(Table.1)

CELL CULTURE, FIXATION AND PERMEABILIZATION

Cervical cancer cell line, Hela cell line is used in this report during system development. Hela cells are grown in MEM medium supplemented with 10% FBA and 0.6% penicillin-streptomycin. After close to a confluence of 90%, suspend cells and transfer cells to a 24-well glass bottom plate with the dilution ratio of 1:8. When the confluency reaches 60%, rinse cells with pre-warmed 1X TBS and fix cells with pre-warmed 4%(wt/vol) paraformaldehyde/TBS for 20 min at 37℃. Rinse with 1X TBS then permeabilize cells in 2%(wt/vol) DTAC/TBS for 20 min at RT. Rinse with 1X TBS then permeabilize cells in 0.25%(wt/vol) Triton X-100/TBS for 5 min at RT. Wash cells three times with 1X TBS at RT and stock cells in 1X TBS in 4℃ for up to several weeks.

DOPAMINE-BIOTIN BASED IF STAINING METHOD IN FIXED CELL SYSTEM

Fixed cells in 24-well plate are incubated in 3% H_2O_2 for 15 min at RT to quench the endogenous peroxidase activity. Rinse cells with 1X TBS then block with various blocking reagents (2% BSA in TBS, 3% goat serum in TBS, Image-iT™ FX Signal Enhancer or blockaid) for 30 min at RT to eliminate non-specific background. Incubate cells with 1st antibody solution (dliluted in Blocking Solution) for 1h at RT. Wash twice with Blocking Solution of choice at RT. Incubate cells with 2nd antibody solution (antibody matching the species of 1st antibody and conjugated with HRP, diluted in Blocking Solution) for various amount of time. Wash twice with Blocking Solution. Add dopamine-biotin assay (20uL dopamine-biotin conjugate solution, 1mL

amplification buffer (500mM borate, 100mM Glycine, pH 8.5), 0.0015% H_2O_2, freshly prepared for each use). Incubate for various amount of time at RT without disturbance. Wash twice with 1X TBS. Incubate with dye-streptavidin conjugate for various amount of time at RT. Wash twice with 1X TBS. Image immediately with cells immerging in 1X TBS. (Table.3, Table.5)

DOPAMINE-FLUOROPHORE BASED IF STAINING METHOD IN FIXED CELL SYSTEM

Fixed cells in 24-well plate are incubated in 3% H_2O_2 for 15 min at RT to quench the endogenous peroxidase activity. Rinse cells with 1X TBS then block with 2% BSA in TBS, 3% goat serum in TBS, Image-iT™ FX Signal Enhancer or Blockaid for 30 min at RT to eliminate non-specific background. Incubate cells with 1st antibody solution (diluted in Blocking Solution of choice) for 1h at RT. Wash twice with Blocking Solution at RT. Incubate cells with 2nd antibody solution (antibody matching the species of primary antibody and conjugated with HRP, diluted in Blocking Solution) for various amount of time. Wash twice with Blocking Solution. Add dopamine-dye assay (various concentration of dopamine-dye conjugate in 1mL amplification buffer (500mM borate, 100mM Glycine, pH 8.5) with 0.0015% H_2O_2, freshly prepared for each use). Incubate for various amount of time at RT and avoid disturbance while incubation. Wash twice with 1X TBS. Image immediately with cells immerging in 1X TBS. (Table.4, Table.6)

SEQUENTIAL MULTICOLOR STAINING IN FIXED CELL SYSTEM

First round of the staining was the same procedure as the one described above for singular staining. After completely washed with 1X TBS, regenerate the cells with IgG Elution

Buffer for 30min at RT and wash several timeswith IgG elution buffer. The efficiency of regeneration was tested with DAB substrate. Reblock cells with Blockaid Blocking Solution and begin next round of staining with same procedure. Image immediately after completing the whole staining procedure with cells immerging in 1X TBS.

SINGLE COLOR STAINING IN FFPE HUMAN TONSIL TISSUE

FFPE human tonsil tissue block was sliced into sections at the thickness of 5um with microtome and placed on charged microscope slides. Sections are dried overnight at 37℃ and store in 4℃for future use.

Tissue sections were deparaffinized with Xylene 3 times 7 min each. After paraffin being completely removed from the slide, slides were rehydrated 100% ethanol 2 times 2 min each, 95% ethanol 2 times 2 min each, 70% ethanol 2 times 2 min each, and 50% ethanol 2 times 2 min each. Wash with DI water for 5 min. Retrieve antigen using Trilogy™ for 15 min with high pressure. Cool down and wash with 1X TBS before staining.

Quench the tissue slides with 3% H_2O_2 /1X TBS for 30 min to prevent endogenous peroxidase . Block tissue with Image-iT™ FX Signal Enhancer for 30 min at RT to eliminate non-specific background fluorescence caused by electrostatic interaction. Rinse cells with 1X TBS then block with Blockaid Blocking Solution for 1h at RT. Incubate tissue with 1st antibody solution (antibody diluted in Blockaid Blocking Solution) overnight at RT. Wash with Blockaid Blocking Solution. Incubate tissue with 2nd antibody solution (antibody matching the species of 1st antibody and conjugated with HRP, diluted in Blockaid Blocking Solution) for various amount of time. Wash twice with Blockaid Blocking Solution.

For dopamine-biotin method, add dopamine-biotin assay (20uL dopamine-biotin conjugate solution, 1mL amplification buffer (500mM borate, 100mM Glycine, pH 8.5), 0.0015%H_2O_2, freshly prepared for each use) and incubate for various amount of time at RT without disturbance. Wash twice with 1X TBS. To test the enzymatic activity of conjugated HRP, DAB substrate was used. Incubate the tissue with fluorophore-streptavidin conjugate for various amount of time at RT. Wash twice with 1X TBS. Mount the slide with anti-fade mounting medium and proceed to image.

For dopamine-dye method, add dopamine-dye assay (various concentration of dopamine-dye conjugate in 1mL amplification buffer (500mM borate, 100mM Glycine, pH 8.5) with 0.0015% H_2O_2, freshly prepared for each use) and incubate for various amount of time at RT without disturbance. Wash twice with 1X TBS. Mount the slide with anti-fade mounting medium and proceed to image.

FLUORESCENCE IMAGING AND MULTISPECTRAL ANALYSIS

X-71 inverted fluorescence microscope (Olympus) equipped with a digital color camera (QColor5, Olympus) and a hyperspectral camera (Nuance, 420–720 nm spectral range) was used for fluorescence imaging. Low-magnification images were obtained with 20X dry objective (Olympus), while high-magnification images were obtained with 40X oil-immersion objective (Olympus). FITC LP filter (460–500 nm band-pass excitation, 510 nm long-pass emission, Chroma) was used for Bella Fluor 488 and FITC. Rhodamine LP filter (530–560 nm band-pass excitation, 572 nm long-pass emission, Chroma) was used for Alexa Fluor 555. Yellow set cube (540–580 nm band-pass excitation, 610 nm long-pass emission, Chroma) was used for Alexa

Fluor 594 and TAMRA. Cy5 LP emission filter (590–650 nm band-pass excitation, 665 nm long-pass emission, Chroma) was used for Alexa Fluor 680.

Nuance image analysis software was used to obtain the reference spectrums of each fluorophore and the autofluorescence spectrum of samples. Unmixing was done based on the spectral library containing all the reference spectrumsin Nuance analysis software. False-color composite images were obtained for the multicolor display.

Confocal image was obtained by Confocal Microscopy (Zeiss 710) equipped with standard PMTs detector from Keck Microscopy Center. Low magnification images were obtained with 20X dry objective, while High-magnification images obtained with 40X oil-immersion objective. Fixed wavelength laser lines, 488nm, 594nm, are used for the excitation of BF488 and BF594, respectively. Image acquirement and analysis was done by Zeiss Zen 2008 software.

QUANTITATIVE ANALYSIS OF FLUORESCENCE IMAGES

All images used for quantitative analysis in this report were acquired by Qcolor5 digital color camera to avoid the instrumental bias caused by hyperspectral system. Relative fluorescence intensity of target was measured by selecting the region of interest and calculate grey value in selected area via ImageJ software. An averaged grey value was used to represent RFU. Similarly. Background fluorescence intensity was measure by calculating grey value in overall cell area. Intensity measurement was only conducted in images with specific staining area. For images without morphological specificity, only qualitative results were shown in this report. Signal to noise ratio (SNR) in this report was calculated by dividing RFU of ROI by RFU of Background. All the charts in this report was plotted in Prism7 software.

Table 1 Conjugation conditions of biotinylated-dopamine and fluorophore-dopamine

Product	Final concentration of NHS-biotin	Final concentration of NHS-Dye	Final concentration of dopamine hydrochloride	Molarity ratio DA: biotin/Dye
Biotin-DA	5mg/mL	-	10mg/mL	3.91:1
BF488-DA	-	5mg/mL	10mg/mL	4:1
BF594-DA	-	5mg/mL	4.5mg/mL	4:1
BF350-DA	-	5mg/mL	15mg/mL	4:1
AF680-DA	-	5mg/mL	3.25mg/mL	4:1
TAMRA-DA	-	5mg/mL	6.6mg/mL	4:1

Table 2 Control design for the study of biotin-DA in fixed cell system

	Primary antibody	Anti-rabbit HRP	Biotin-DA	Fluorophore-SA
Condition 1: Staining for specific targets	+	+	+	+
Condition 2: Control for effects of 2nd Abs	-	+	+	+
Condition 3: Control for effects of biotin-DA	-	-	+	-
Condition 4: Control for effects of Fluorophore-SA	-	-	-	+

Table 3 Control design for the study of fluorophore-SA in fixed cell system

	Primary antibody	Anti-rabbit HRP	Fluorophore-DA
Condition 1: Staining for specific targets	+	+	+
Condition 2: Control for effects of 2nd Abs	-	+	+
Condition 3: Control for effects of Fluorophore-DA	-	-	+

Table 4 Conditions for the study of biotin-DA in fixed cell system

	Blocking method	Primary Abs	Secondary Abs	Biotin-DA	Fluorophore-SA
Original conditions	2% BSA	Rabbit anti-HSP90 • 10ug/mL • 1h	Goat anti-rabbit HRP • 10ug/mL • 1h	• 100ug/mL • 50min	FITC-SA • 20ug/mL • 5min
					AF555-SA • 40ug/mL • 5min
		Rabbit anti-LaminA • 2ug/mL • 1h			FITC-SA • 20ug/mL • 5min
					AF555-SA • 40ug/mL • 5min
Optimized condition	Blockaid	Rabbit anti-HSP90 • 10ug/mL • 1h	Goat anti-rabbit HRP • 5ug/mL • 10min	• 100ug/mL • 30min	FITC-SA • 1ug/mL • 5min
		Rabbit anti-LaminA • 2ug/mL • 1h			

Table 5 Conditions for the study of Fluorophore-DA in fixed cell system

	Blocking method	Primary Abs	Secondary Abs	Fluorophore-DA
Original conditions	Blockaid	Rabbit anti-HSP90 • 10ug/mL • 1h Rabbit anti-LaminA • 2ug/mL • 1h	Goat anti-rabbit HRP • 5ug/mL • 1h	AF594-DA • 10ug/mL • 30min
Optimized condition	Blockaid	Rabbit anti-LaminA • 2ug/mL • 1h	Goat anti-rabbit HRP • 5ug/mL • 30min	AF594-DA • 10ug/mL • 5min

Table 6 Conditions for the study of biotin-DA, Fluorophore-DA in FFPE tissue

	Blocking method	Primary Abs	Secondary Abs	Biotin-DA	Fluorophore-SA	Fluorophore-DA
Biotin-DA & Fluorophore -SA system	3% goat serum + ABC biotin blocking	Mouse anti-CD20 • 1:1000 • Overnight	Goat anti-mouse HRP • 4ug/mL • 1h	• 100ug/mL • 1h	• 20ug/mL • 30min	-
Fluorophore -DA system	Blockaid + Image Fx Enhancer	Mouse anti-CD20 • 1:1000 • Overnight	Goat anti-mouse HRP • 10ug/mL • 1h	-	-	AF594-DA • 10ug/mL • 30min

RESULTS AND DISCUSSION

BIOTIN-DOPAMINE STAINING SYSTEM IN FIXED CELLS

The biotin-dopamine method utilized HRP to catalyze the polymerization of dopamine and provided biotin-site for the attachment of fluorophore-SA. The original staining experiment showed large amount of background caused by non-specific binding of secondary antibody (Figure.5a) and non-neglectable background caused by endogenous peroxidase (Figure.5c, middle). Those results provided some insights for the later-on optimization: (1) 2^{nd} antibody is a critical component to be considered to improve the overall performance; (2) more efficient inactivation of endogenous peroxidase should be included in pre-staining procedure. After optimizing with several critical components in the system: 2^{nd} antibody, biotin-DA, fluorophore-SA and blocking method, we improved the overall performance of biotin-dopamine method. Compared to the result of initial staining, the signal to noise ratio increased in LaminA channel even though the overall intensity decreased after optimization. However, in HSP90 channel, the SNR even decreased after the optimization, which could be explained by the difference of assay sensitivity in various targets. (Figure.6)

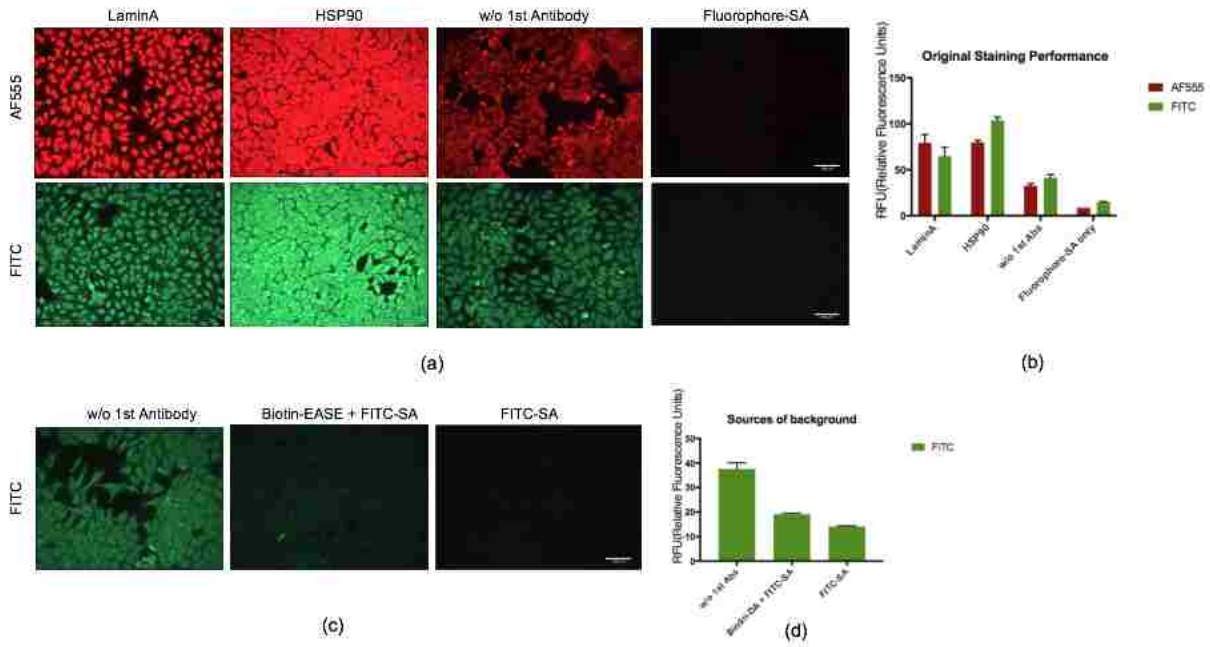

Figure 5 Original staining performance and sources of background via biotin-dopamine
(a) Performance of biotin-dopamine system before optimization in FITC-SA (bottom panel, green channel) and AF555 (top panel, red channel). (b) Quantification of fluorescence intensity in different channels from (a). (c) Experiment identifying sources of background via different controls: effect of 2nd antibody (left), effect of biotin-dopamine (middle) and effect of fluorophore-SA alone (right). (d) Quantification of fluorescence intensity in (d). Scale bar 100um.

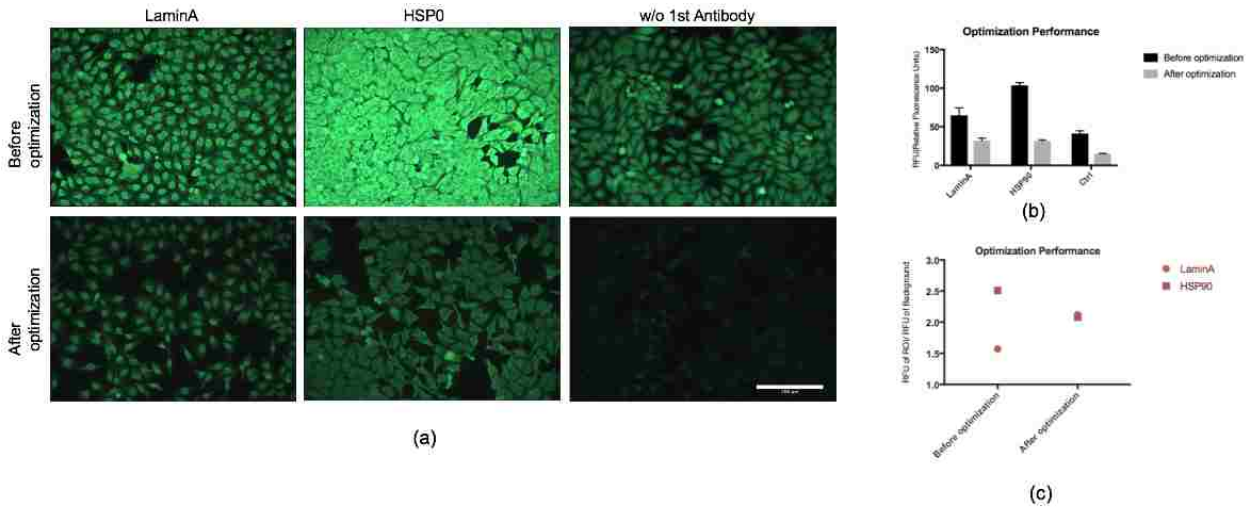

Figure 6 Comparison of performance between un-optimized and optimized condition

(a)Fluorescence images of cells stained under: un-optimized condition (top panel), optimized condition (bottom panel). Quantification of fluorescence intensity in (b), signal to noise ratio in (c). Scale bar 200um.

DEVELOPMENT OF FLUOROPHORE-DOPAMINE STAINING SYSTEM IN FIXED CELLS

Similarly, the fluorophore-DA staining system is relying on the enzymatic reactivity of HRP to polymerize of DA in proximity of HRP, meanwhile, deposit fluorophore in a spatially specific manner. The initial staining result before optimization showed non-neglectable amount of background signal in control groups (Figure.7a, 7b). Different from the biotin-DA system, the main source of background signal was caused by the non-specific binding of fluorophore-DA (Figure. 7c, 7d) instead of 2^{nd} antibody. This result demonstrated the importance of eliminating fluorophore-DA non-specific interaction in later optimization. Two main aspects have been considered for optimization: 2^{nd} antibody and fluorophore- DA. The optimization improved the overall performance of staining, which is reflected by the increased SNR without sacrificing the absolute fluorescence intensity of targets. We then applied different types of fluorescent dye visualizing multiple distinctive subcellular targets under optimal condition (Figure.8).

We also observed that due to the difference in chemical structure and affinity to cellular compartments, different fluorophores caused different level of background signal under the same staining condition. According to our results, AF594 caused most background signal in control group, while AF 350 had least background signal. Therefore, the choice of fluorophore with low affinity to non-specific subcellular structures is also quite necessary.

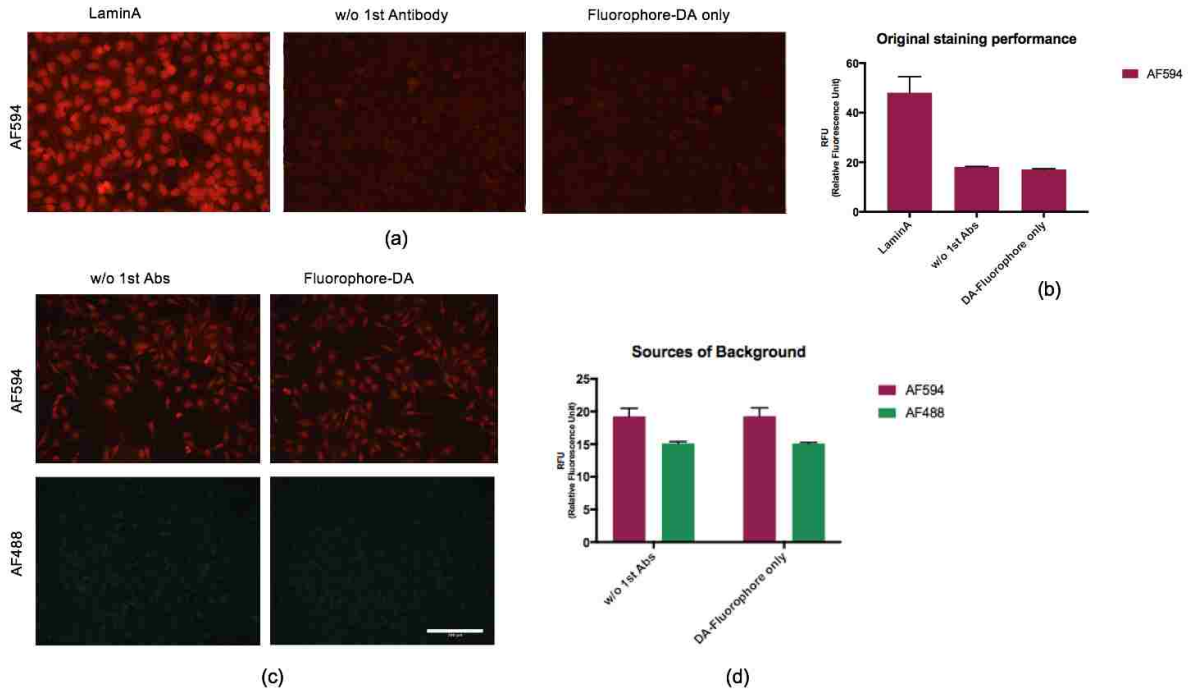

Figure 7 Original staining performance and sources of background signal in fluorophore-dopamine system

(a) Staining performance of AF594-dopamine system before optimization. (b) Quantification of fluorescence intensity in (a). (c) Experiment identifying sources of background via different types of controls: effect of 2nd antibody (left) and effect of fluorophore-dopamine (right) in AF594 (top panel) and AF488 (bottom panel). (d) Quantification of fluorescence intensity in (c). Scale bar 200um.

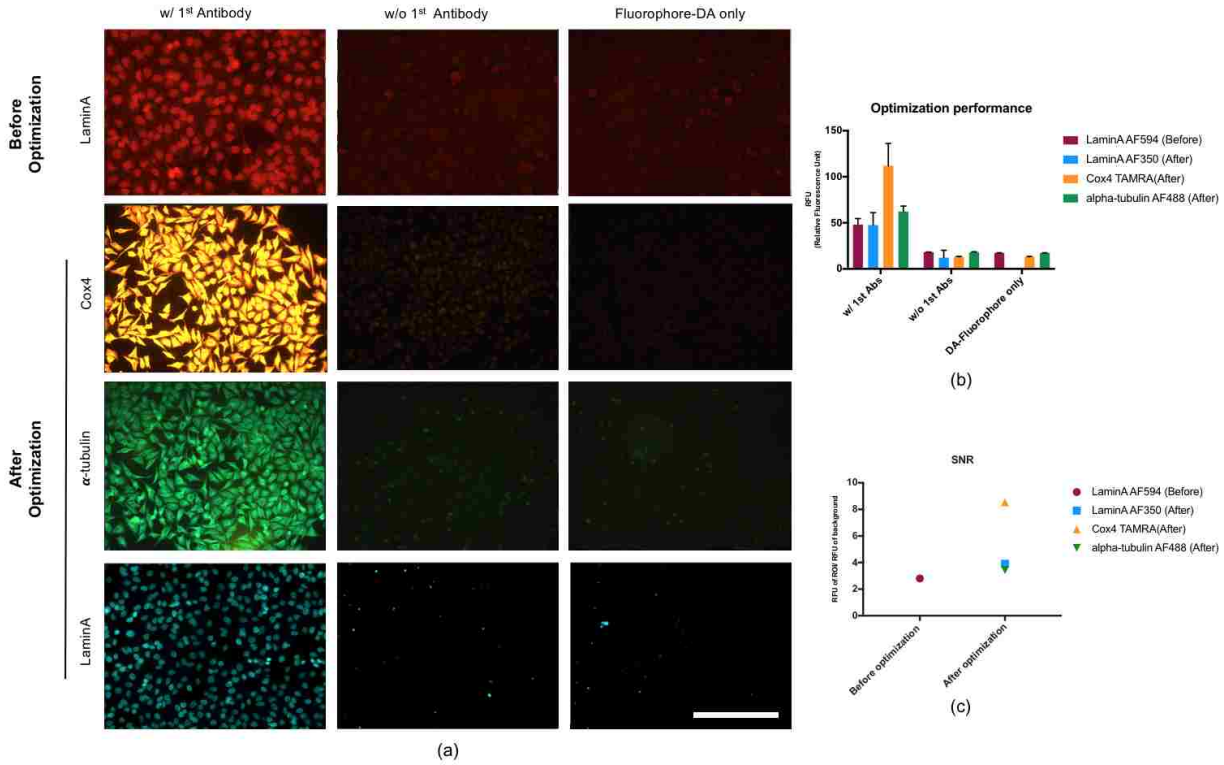

Figure 8 Comparison of staining performance between un-optimized condition and optimized condition in fluorophore-DA system

(a)Fluorescence images of cells stained with: un-optimized condition (top panel), optimized condition (bottom panel) with different dyes and targets. Quantification of fluorescence intensity in (b), signal to noise ratio in (c). Scale bar 100um.

MULTICOLOR MULTISPECTRAL IMMUNOFLUORESCENCE IN FLUOROPHORE-DOPAMINE SYSTEM

When developing a multiplexing imaging platform, the first consideration is how to regenerate the sample to allow multiple rounds of labeling on the same sample. There are multiple strategies to achieve this goal: (1) inactivate the fluorophore and cancel out the fluorescence after each round of staining; (2) quench the signal-generating component after each round; (3) completely remove the antibody-complex from the system. Since our method of fluorophore-DA relies on enzymatic reactivity to develop the fluorescence, quenching the HRP after each round or removing antibody-HRP complex could both be efficient for regeneration. We first tested various HRP quenching methods in a single-color staining model, in which the more efficient the quenching is, the less fluorescence would be developed in the system (Figure.9a, 9d). Our result showed that both HCl and H2O2 had capacity to quench HRP compared to control, but detectable signal was still observed after quenching. Then we tested another method using acidic IgG elution buffer, which could both quench HRP with low pH and remove antibody complex by dissociating antibody-antigen interaction. Our result indicated that after treated with IgG elution buffer, there's no detectable HRP activity shown by DAB substrate, which suggested an efficient elution result for another round of labeling (Figure.9b, 9e). Additionally, we also tested the effect of elution step on previously developed fluorescence signal and found no significant change in intensity after elution (Figure.9c,9f).

Comparing the staining results of biotin-DA system and fluorophore-DA system, we concluded that the overall performance was more promising in the fluorophore-DA system based on the optimization result. Therefore, we developed multi-color staining method based on fluorophore-DA system (Figure.4). The workflow of this method includes applying antibodies

upon the targets, developing fluorescence with DA-fluorophore substrate, removing antibody-antigen complex with regeneration buffer leaving deposited fluorophore in the proximity of target and entering another staining cycle by applying another round of blocking and antibodies. The image acquisition was conducted after completing fluorescence for all targets. Multiple combinations of fluorophores were used in multicolor staining system to obtain optimal staining result. To solve the emission overlapping problem when multiple fluorophores with close emission wavelengths used in the same combination, multispectral imaging platform was introduced into the workflow. Additionally, the multispectral imaging could also remove autofluorescence signal from the sample. We first validated and developed the multispectral imaging protocol in single-color samples (Figure.10). We observed an increase in specific signal after unmixing based on correspondent reference spectrums. Interestingly, when exciting AF350 with short wavelength laser light, more autofluorescence was observed in the sample (Figure.9a, bottom left) and the multispectral system was able to identify and eliminate autofluorescence (Figure.9a, bottom right). Similarly, the unmixing procedure also worked in tissue sample by boosting the specific signal and eliminating unwanted background caused by autofluorescence in tissue samples. Based on results described above, we then developed two-color staining with DA-fluorophore system and unmixed signals from different fluorophores via multispectral platform established above (Figure.11)

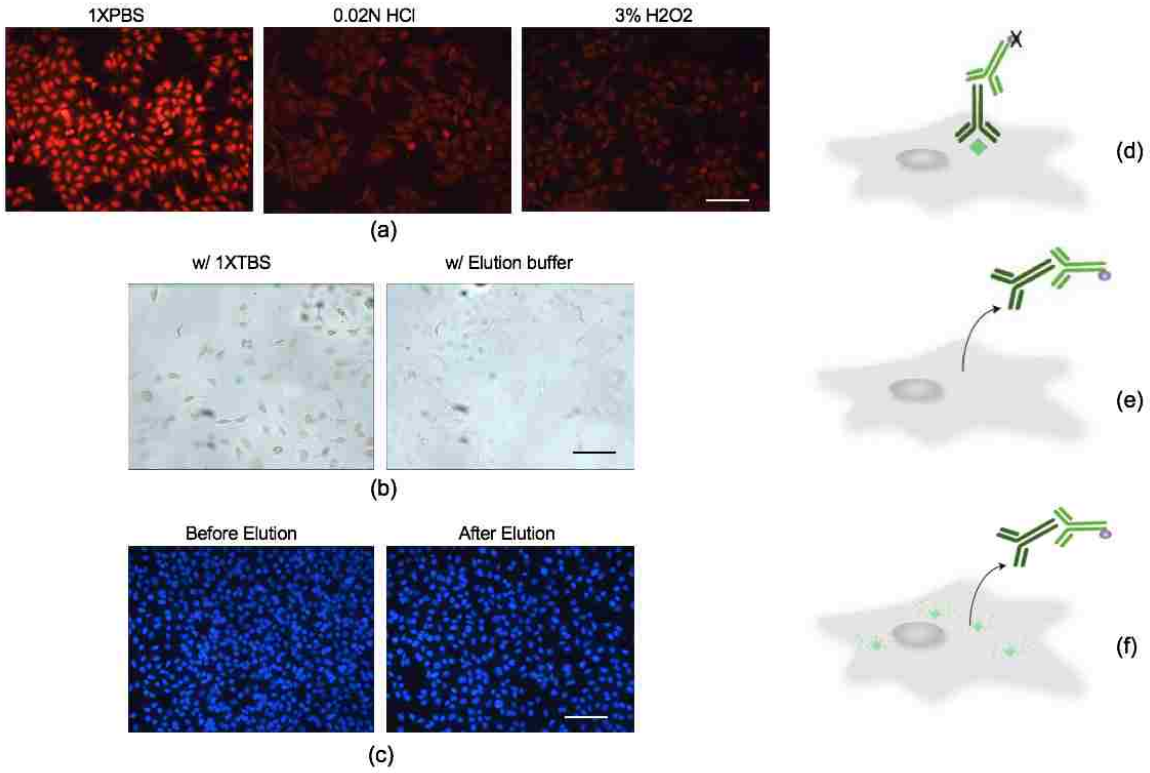

Figure 9 Study of regeneration method for multi-color staining in fluorophore-DA system

(a) Different methods to quench HRP conjugated on 2nd antibody: 1XPBS (control, left panel), 0.02N HCl (middle panel), 3%H2O2 (right panel); and the scheme of quenching mechanism (d). (b) Regeneration efficiency of IgG Elution Buffer: 1XTBS (Control, left panel), IgG elution buffer (right panel); and the scheme for elution mechanism (e). (c) Effect of regeneration procedure on fluorescence intensity and correspondent scheme (f). Scale bar 100um.

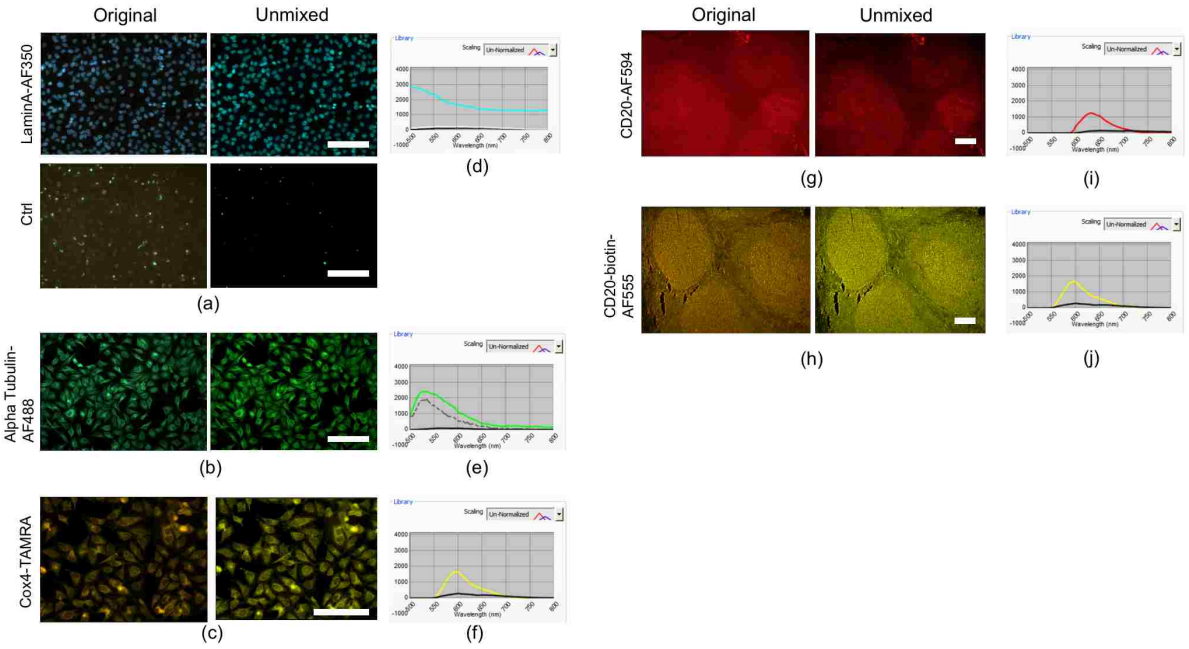

Figure 10 Study of multispectral imaging in single-color fluorophore-DA system

(a)Multispectral analysis result of LaminA labelled with DA-AF350 and the correspondent spectral library (AF350, cyan line; autofluorescence, black line) used for unmixing (d). (b)Multispectral imaging and analysis result of Alpha-Tubulin labelled with DA-AF488 and the correspondent spectral library (AF488, green line; autofluorescence, black line) used for unmixing (e). (c)Multispectral imaging and analysis result of Cox4 labelled with DA-TAMRA and the correspondent spectral library (TAMRA, yellow line; autofluorescence, black line) used for unmixing (f). (g)Multispectral imaging and analysis result of CD20 labelled with DA-AF594 in tonsil tissue and the correspondent spectral library (AF594, red line; autofluorescence, black line) used for unmixing (i). (h)Multispectral imaging and analysis result of CD20 labelled with DA-biotin and AF555-SA in tonsil tissue and the correspondent spectral library (AF555, yellow line; autofluorescence, black line) used for unmixing (j). Scale bar 100um.

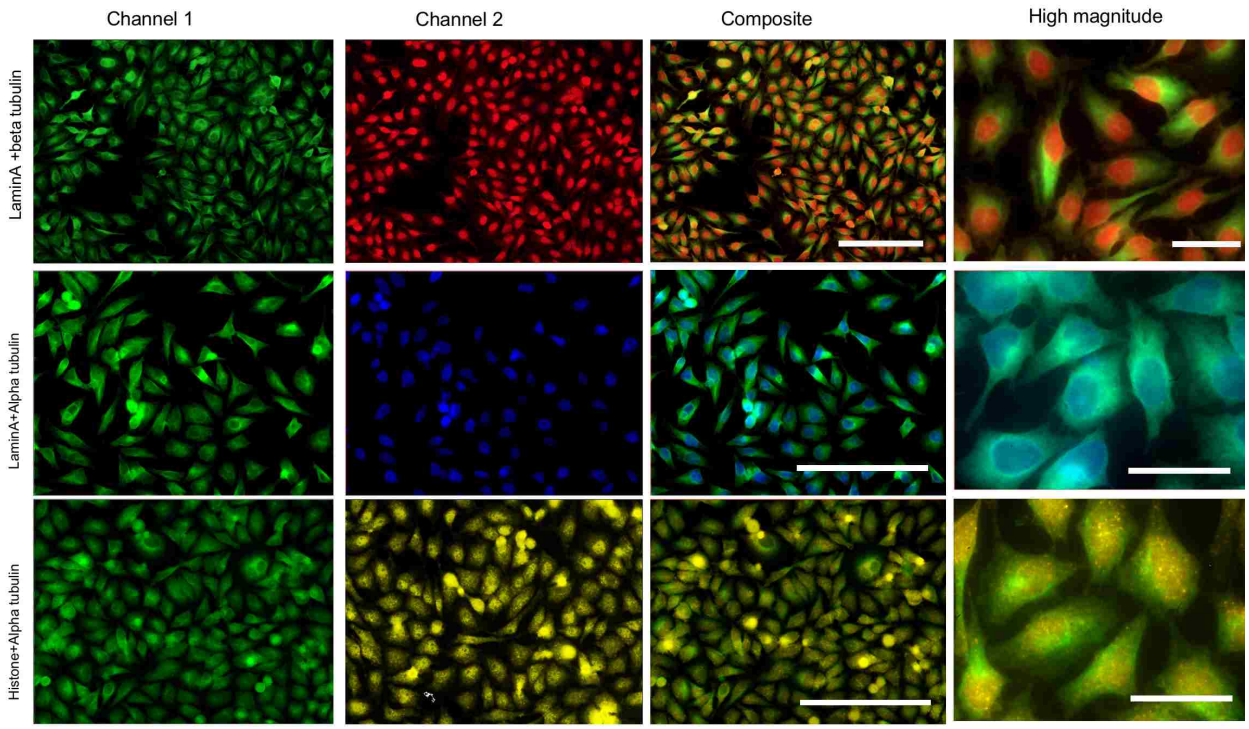

Figure 11 Study of multi-cycle multicolor staining in fluorophore-DA system

1st row: two-color staining of beta-tubulin (green, AF488) and LaminA (red, AF594) with composite image (3nd panel) and zoom-in image (4th panel, 40X oil immersion).

2nd row: two-color staining of alpha-tubulin (green, AF488) and LaminA (blue, AF350) with composite image (3nd panel) and zoom-in image (4th panel, 64X oil immersion)

3rd row: two-color staining of alpha-tubulin (green, AF488) and histone H3 (yellow, TAMRA) with composite image (3nd panel) and zoom-in image (4th panel, 64X oil immersion)

Images all capture by Nuance multispectral imaging system and unmixed based on correspondent reference library. Scale bar for first three panel 100um, scale bar for last panel 50um.

APPLICATION OF DOPAMINE-BASED IMMUNOFLUORESCENCE STAINING IN FFPE TISSUE

The final goal of this project is to develop a novel amplification method for rapid immunofluorescence detection using tissue biopsy. Therefore, we then tested the compatibility of our staining systems in FFPE tonsil tissue sample. The overall procedure for tissue staining was similar to the one we established in fixed cell system, expect for some changes in staining condition. In FFPE tonsil tissue, CD20, a B-lymphocyte antigen, was labelled, by biotin-DA and fluorophore-DA and a clear structure of lymphatic nodules, where B cells reside and undergo a maturation process, was observed after staining. Compared to the traditional IHC method using DAB substrate (Figure.12a, top row), our method demonstrated better resolution on desired structure (Figure.12a, middle and bottom row), although there's still significant background observed in the biotin-DA system, even under confocal microscopy (Figure.12b).

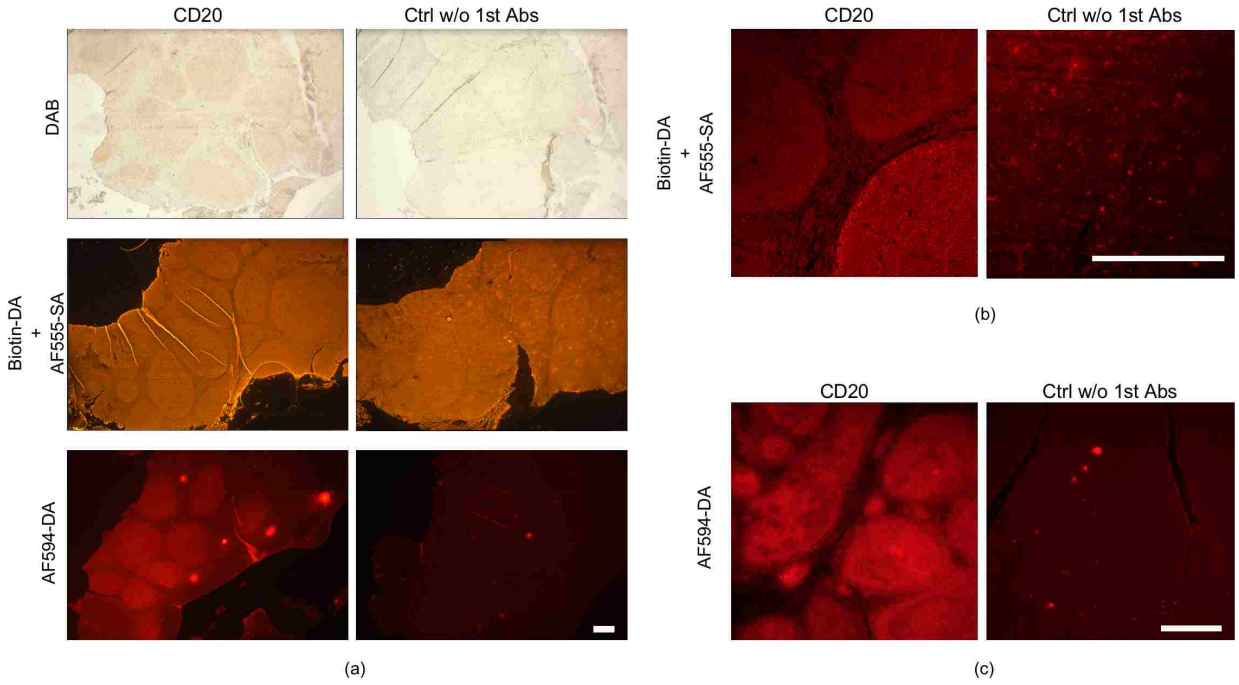

Figure 12 Immunohistochemistry and immunofluorescence staining of FFPE tonsil tissue
(a) Staining results of traditional IHC with DAB substrate (top row), biotin-DA system (middle row) and fluorophore-DA system (bottom row). (b) Confocal image of tonsil tissue stained with biotin-DA system. (c) Zoom-in image of tonsil tissue stained with fluorophore-DA system. Scale bar 300um.

CONCLUSIONS AND FUTURE DIRECTIONS

In summary, the work described in this report demonstrate a simple immunofluorescence method with potential for signal amplification and multiplexing imaging based on the polymerization reaction of polydopamine catalyzed by HRP. Two different strategies introduced in this document, biotin-DA and fluorophore-DA systems, both succeeded in developing fluorescent signal with specificity to target of interest, while the fluorophore-DA system has better performance in amplifying the signal, especially increasing SNR after optimization both in fixed cells and FFPE tissue. Finally, multi-color staining is enabled by fluorophore-DA system in the fixed cells along the assistance of multispectral imaging system. Overall, the work described in this report provides another possible solution for signal amplification in the diagnostic context.

More efforts need to be made to further expand the multiplexing capacity of fluorophore-DA system. Main obstacle we faced in developing multi-color staining system was the accumulation of background signal after each round of labeling, which limited the numbers of target we can label in one sequential. Therefore, an in-depth study of the cause of the background signal as well as the physicochemical property of dye molecules are also necessary to maximally avoid unwanted noise signal in a highly multiplexed setting. It was previously described that hydrophobicity was a key determinant influencing non-specific binding of fluorophores onto substrate[20].

Further, we only established our multi-color workflow within one sequential in this report, which limited the number of target we can measure in single specimen. When all the available subcellular space is occupied by fluorescence, the study of removal/bleaching method for fluorophores is also necessary to further improve multiplexing capacity. Alkaline oxidation chemistry was previously reported as an inactivation solution for cyanine-based fluorescent dye,

which could be another option for dye selection in multiplexed analysis [21]. Another factor that limited multiplexing capacity is the photophysical properties of dye molecule. The fluorescent dyes we used in this report all have broad-emission spectrums that cause inevitable fluorescence leakage between channels. Although multispectral imaging system was introduced to solve the problem, to maintain the accuracy of quantitative analysis in multiplexed images, fluorescent dye with narrow-emission spectra needs to be taken into consideration. Previously study in our demonstrated the feasibility and multiplexibility of quantum dots in single-cell molecular profiling due to its special excitation-emission property [22].

As previously mentioned, the final aim of this project is to achieve an efficient diagnostic platform for tissue biopsy. Thus, other than improving multiplexibility of the platform, another direction is to shorten the overall assay time. The fluorophore-DA system has already emerged its potency in rapid development, which could generate sufficient amount of fluorescent signal in fixed cells in 5min. If this feature could be adapted onto tissue specimen, a real-time bedside specimen analysis method could be developed, implementing a timely monitoring of cancer patients beared with rapidly-growing aggressive tumor.

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