

# Creating Complex Fluorophore Spectra on Antibodies Through Combinatorial Labeling

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## ABSTRACT

Fluorescently-labeled antibodies are central to many biochemical assays, but they are not easy to multiplex beyond 3-4 colors. A long-term hypothesis of ours is that labeling antibodies with multiple fluorophores, in a way such that fluorescence resonance energy transfer (FRET) occurs, may provide a way to increase fluorescence multiplexing ability by creating a rich variety of complex emission spectra that could be deconvolved via spectral methods. However, it is not yet clear how one can effectively label antibodies with multiple fluorophores that exhibit FRET. Here, we show how to use Mix-n-Stain antibody labeling kits from Biotium to label antibodies with multiple fluorophores that exhibit FRET. Key to our approach is the use of Fab fragments, as opposed to full IgG molecules, since the full IgG molecules are generally too large to allow the fluorophore proximity necessary for observable FRET. We show that our approach works with two different sets of FRET-capable fluorophore combinations: CF405A/CF488M and CF568/CF640R. These results form the basis for continued development of approaches for increased multiplexing of fluorescent antibody measurements.

**Citation:** Holzapfel HY, Birtwistle MR (2016) Creating Complex Fluorophore Spectra on Antibodies Through Combinatorial Labeling. *Transl Science* 2(3):e03

**Academic Editor:** Dat Q. Tran | UTHealth McGovern Medical School | Houston, Texas, USA.

**Reviewer #1:** Shuja S. Malik | **Reviewer #2:** undisclosed

**Received:** 02/19/2016 | **Published:** 03/31/2016

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**Competing Interests:** The authors have declared that no competing interests exist.

**Funding:** This study was supported by funds from TranScience Student Mentor Academic Research Training (SMART) Award and a grant from the National Institutes for Health (1R21CA196418-01).

**Author Contributions:** HYH designed and performed the experiments and wrote the manuscript. MRB designed the experiments and edited the manuscript.

**Acknowledgement:** Thank you to Dr. Lori Roberts and Dr. Wai-Yee Leung from Biotium for their helpful discussions.

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## INTRODUCTION

Advances in multiplexing technologies such as deep sequencing have transformed the way we can probe tumor biopsy samples for biomarkers indicative of prognosis and treatment response. Routine yet arguably more clinically relevant staining analyses of tumor sections reveal important *in situ* information not easily obtainable by such highly multiplexed methods, but staining analyses are not highly multiplexed and typically remain limited to ~4-5 analytes, or 7 with multi-spectral imaging [1]. Recent technologies have made strides in this direction, such as mass cytometry to multiplex 32 mass-tagged antibody measurements from tumor sections [2], super-resolution imaging combined with *in situ* hybridization and combinatorial labeling to measure 32 nucleic acids in single yeast cells [3], and cycles of staining with chemical inactivation to analyze 61 antigens in tumor sections [4,5]. However, these techniques require expensive equipment and/or reagents, sophisticated analyses or markedly increased assay time, all of which would preclude their practical use in many clinical pathology and preclinical research laboratories. Thus, there is a significant need for technologies that multiplex antibody-based measurements *in situ* but are also widely accessible and cost-effective. One potential way to increase fluorescent antibody multiplexing is to label primary antibodies not only with a single fluorophore, but also with multiple fluorophores simultaneously, in a way that fluorescence resonance energy transfer (FRET) occurs to create new, multi-modal emission spectra. This goal is the purpose of the current study. Multiple labeling of antibodies in a flexible and tunable way has not been done before to our knowledge; thus, we are piloting a novel technique. We used the Biotium CF405M Mix-N-Stain and CF488A Mix-N-Stain kits to label one antibody with CF405M, one antibody with CF488A, and a third antibody with both CF405M and CF488A on the same molecule. During our experiments we found a whole IgG molecule to be too large to allow FRET to occur, so we applied our method to Fab fragments which resulted in FRET on the dual-labeled antibodies. We found that another fluorophore combination (CF568 Mix-N-Stain and CF640R Mix-N-Stain kits) also led to FRET on dual-labeled Fab fragments. This method is in principal readily adoptable to many clinical pathology and preclinical research laboratories.

## METHODOLOGY

### Antibodies

Non-specific antibodies obtained were normal rabbit IgG (Cat #: NI01-100UG, Lot: D00168753, Calbiochem, EMD Millipore

Corp., Billerica, MA) and rabbit IgG Fab fragment (Cat #: 011-01050002, Lot: 33009, Rockland Immunochemicals Inc., Limerick, PA). Both antibodies were diluted to a concentration of 1.0 mg/mL with PBS.

### Mix-n-Stain Antibody Labeling

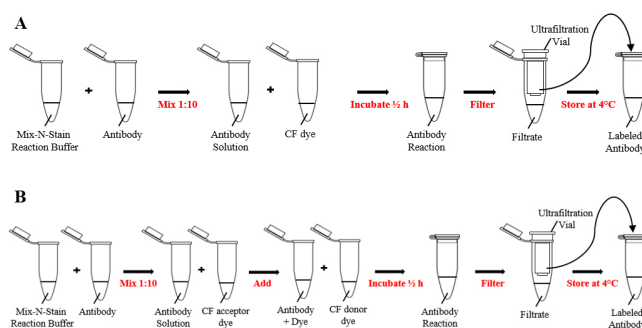
The Mix-n-Stain CF Dye Antibody Labeling Kits were obtained from Biotium Inc. (Mix-n-Stain CF405M Antibody Labeling Kit Cat #: 92272; Mix-n-Stain CF488A Antibody Labeling Kit Cat #: 92273; Mix-n-Stain CF568 Antibody Labeling Kit Cat #: 92275; Mix-n-Stain CF640R Antibody Labeling Kit Cat #: 92278). The manufacturer's protocol was generally followed as described in the following and Figures 1A-B. The Mix-n-Stain Reaction Buffer vial and the Mix-N-Stain Storage Buffer vial were warmed to room temperature before use. The vials were briefly centrifuged. One  $\mu\text{L}$  of the 10X Mix-n-Stain Reaction Buffer was added to 9  $\mu\text{L}$  antibody solution (1.0 mg/mL). The solutions were mixed by pipetting up and down and then transferred to the vial containing the CF dye. For dual labeling, the solution was transferred to the acceptor (red-shifted) dye first and thoroughly mixed by pipetting up and down, and then, after 10 minutes, the solution was transferred to the donor (blue-shifted) dye. For both single and dual labeling, the vial was then vortexed for a few seconds and incubated in the dark at room temperature for 30 minutes. The solution was transferred to the membrane of an ultrafiltration vial (Biotium Lot# 13551746, MW cutoff = 10 kDa), and centrifuged at 14,000  $\times g$  for 4 minutes, or longer until all of the liquid was filtered into the receiving vial. The membrane was washed 3 times with 300  $\mu\text{L}$  PBS at 14,000  $\times g$  for 6 minutes each time. The antibody-dye conjugate on the membrane was re-suspended in 60  $\mu\text{L}$  Mix-N-Stain Storage Buffer and transferred to a microcentrifuge tube wrapped in aluminum foil for storage at 4°C.

### Spectral Scanning

The spectra of the antibody-dye solutions were measured on a Shimadzu RF-5301PC spectrofluorometer. One  $\mu\text{L}$  of antibody-dye solution was diluted in 699  $\mu\text{L}$  PBS in a 700  $\mu\text{L}$  quartz cuvette (Thor Labs). The CF405M and CF488A dyes were excited at 390 nm and 460 nm, and the CF568 and CF640R dyes were excited at 540 nm and 620 nm. A 2D emission spectral scan was taken of each sample with an emission start 15 nm after the excitation channel and an emission end at 700 nm. The instrument parameters were set to high sensitivity with an excitation slit width of 5 nm and an emission slit width of 10 nm. Scanning parameters were set to very fast scanning speed, auto response time and 1 nm intervals. Each sample was measured in triplicate.

### Calculations

All data were imported into MATLAB. The data of each sample were stacked into one column but separated by excitation channel. First, the average of each sample measured in triplicate was taken and then each value within a column was divided by the maximum value measured for a group (e.g. CF405M, CF488A, and CF405M-CF488A). The resulting value was termed 'Relative Intensity'. To compare spectra for evidence of FRET, the 'Integral Normalized Intensity' was taken by dividing each value in a column by the sum of the column.



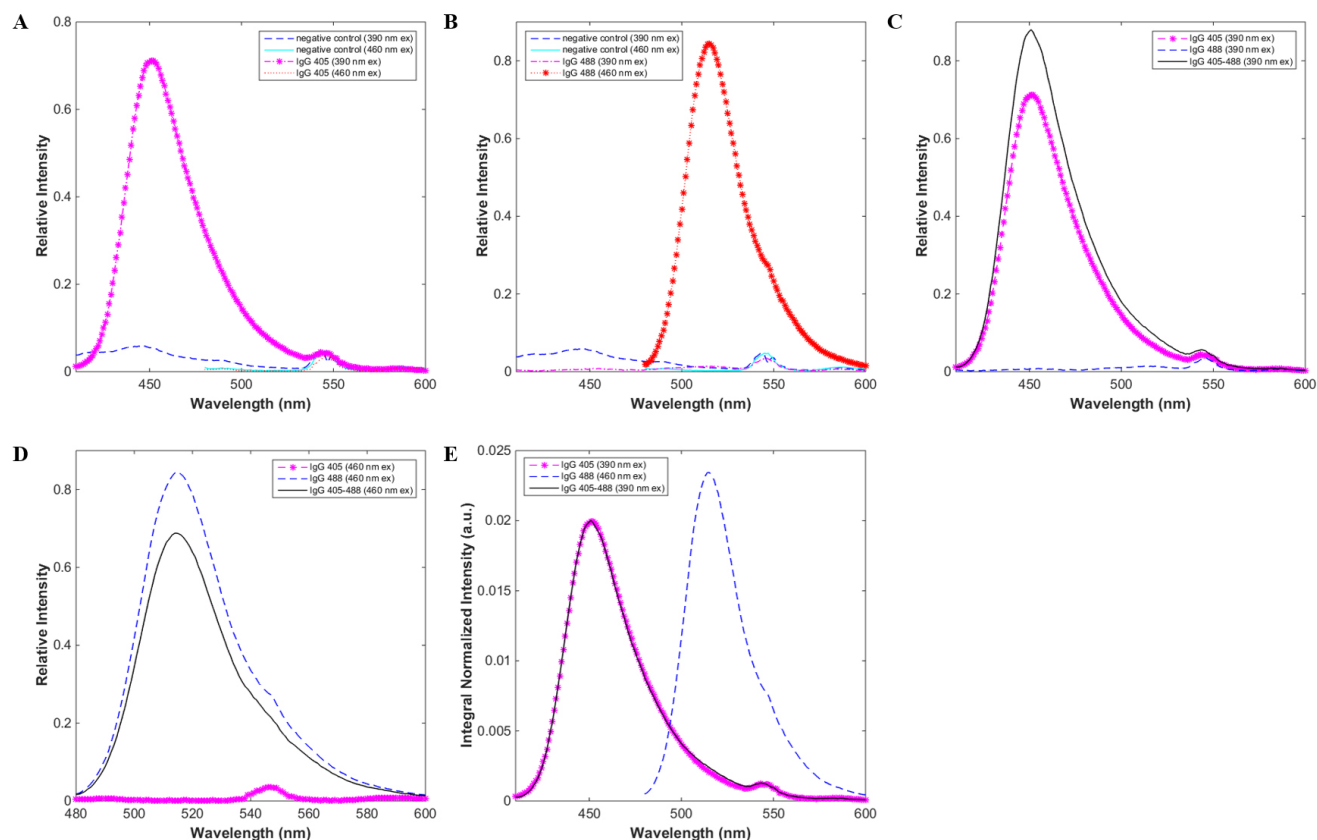
**Figure 1 | Experimental flowchart for labeling antibodies with Mix-n-Stain kits. (A)** The flowchart represents the single-label process for antibodies using a Mix-n-Stain kit. **(B)** The flowchart visualizes the dual-labeling of antibodies using two Mix-n-Stain kits.

## RESULTS

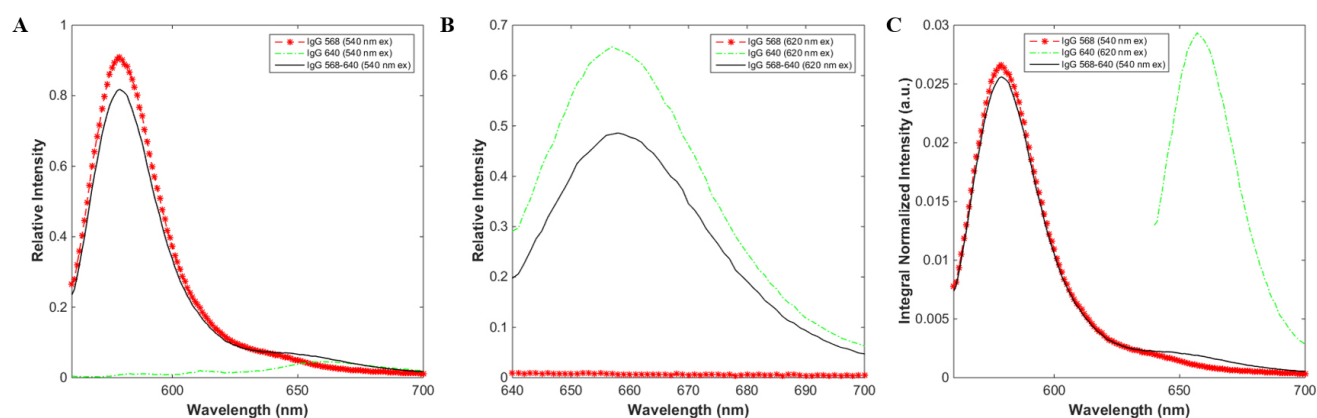
### Single and Double Labeling of IgG

The Mix-n-Stain antibody labeling protocol (Fig. 1) was first applied to normal rabbit IgG using the CF405M and CF488A dye kits, either alone, or using the dual-labeling approach (Fig. 2). CF405M and CF488A were chosen because their excitation and emission spectra should allow for significant potential FRET. A negative control was included by following the labeling protocol using a CF405M and CF488A dye kit without adding IgG antibodies, to evaluate whether observable levels of free dye remain after the labeling process. The negative controls have negligible background signal compared to the labeled antibodies. The CF405M-labeled antibody has an emission peak at ~450 nm in the 390 nm excitation channel (Fig. 2A) and the CF488A-antibody has a peak at ~515 nm in the 460 nm excitation channel (Fig. 2B), as expected. The CF488A-labeled antibodies have negligible emission intensity after 390 nm excitation (Fig. 2C), and the CF405M-labeled antibodies have negligible emission intensity after 460 nm excitation (Fig. 2D), demonstrating the 390 nm and 460 nm excitation wavelengths are reasonably specific for CF405M and CF488A, respectively. In comparison, the dual-labeled CF405M-CF488A antibodies show emission spectra at both excitation wavelengths (Fig. 2C-D) demonstrating that the dual antibody labeling was successful in conjugating both CF405M and CF488A. However, the emission spectra for the dual-labeled CF405M-CF488A IgG at 390 nm excitation did not exhibit a significant difference in shape as compared to the CF405M IgG alone (Fig. 2E), as would be expected if CF405M were exhibiting FRET to CF488A on the dual-labeled antibodies. We conclude that while the dual-labeling protocol does succeed in putting both fluorophores onto the antibodies, there is not significant FRET.

The Mix-n-Stain antibody labeling protocol was also applied to normal rabbit IgG using the CF568 and CF640R dye kits (also expected to exhibit FRET). Labeling was successful with CF568-labeled antibodies exhibiting an emission peak at ~580 nm in the 540 nm excitation channel (Fig. 3A) and CF640R-labeled antibodies at ~655 nm in the 620 nm excitation channel (Fig. 3B). The CF568-labeled antibodies have negligible



**Figure 2 | Labeling normal rabbit IgG with CF405M and CF488A Mix-n-Stain kits with negative controls.** All spectra are measured in triplicate. **(A)** CF405M-labeled IgG compared to a CF405M negative control (no IgG), both excited at 390 nm and 460 nm. **(B)** CF488A-labeled IgG compared to a CF488A negative control (no IgG), both excited at 390 nm and 460 nm. **(C)** 390 nm excitation of CF405M-, CF488A-, and CF405M-CF488A-labeled antibodies. **(D)** 460 nm excitation of CF405M-, CF488A-, and CF405M-CF488A-labeled antibodies. **(E)** CF405M-, CF488A-, and CF405M-CF488A-labeled antibodies at their optimal excitation wavelengths. Note: The y-axis here is integral normalized to make FRET (or lack thereof) apparent by comparing spectra to one another.



**Figure 3 | Labeling normal rabbit IgG with CF568 and CF640R Mix-n-Stain kits.** All spectra are measured in triplicate. **(A)** 540 nm excitation of CF568-, CF640R-, and CF568-CF640R-labeled antibodies. **(B)** 620 nm excitation of CF568-, CF640R-, and CF568-CF640R-labeled antibodies. **(C)** CF568-, CF640R-, and CF568-CF640R-labeled antibodies at their optimal excitation wavelengths. Note: The y-axis here is integral normalized to make FRET (or lack thereof) apparent by comparing spectra to one another.

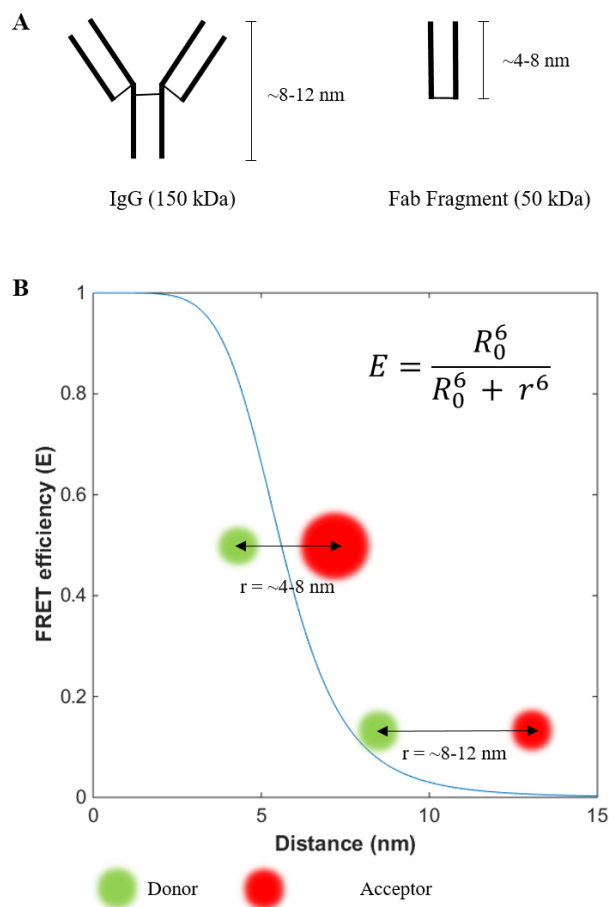
emission intensity after 620 nm excitation, as do the CF640R-labeled antibodies after 540 nm excitation. This shows that the 540 nm and 620 nm excitation channels are reasonably specific for CF568 and CF640R, respectively (similar to above). The antibodies incubated with both CF568 and CF640R display the expected emission peaks in both excitation channels, again indicating successful dual labeling (Fig. 3A-B). However, similar to the CF405M-CF488A results above, there was no evidence for observable FRET between CF568 and CF640R on dual-labeled IgG.

### Single and Double Labeling of Fab Fragments

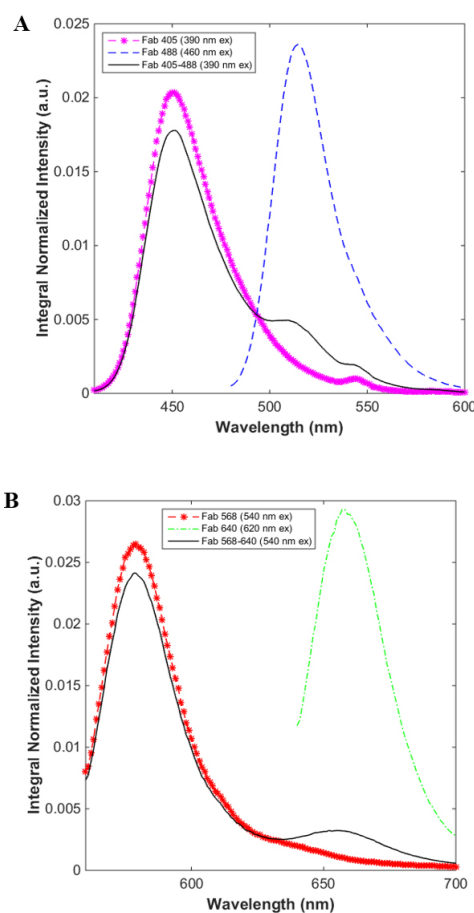
FRET between two fluorophores is extremely sensitive to their physical proximity (Fig. 4). A FRET pair is made up of two fluorophores, one acting as the donor and the other as the acceptor. FRET efficiency ( $E$ ) can be calculated based on the equation  $E = (R_0^6)/(R_0^6 + r^6)$ , where  $R_0$  is the Förster distance between the fluorophores at which  $E=50\%$  and  $r$  is the distance between the two fluorophores. Typical  $R_0$  values for such visible

light fluorophores are typically in the range of  $\sim 5$ -8 nm [6]. Thus, with small molecule fluorophores such as the CF dyes, there is a  $\sim 50\%$  FRET efficiency if the donor and acceptor fluorophores are  $\sim 5$  nm apart. Diameter of an IgG molecule is  $\sim 8$ -12 nm. Between a distance of 8-12 nm FRET efficiency is below 10%, and therefore two fluorophores on a single IgG molecule are highly unlikely to exhibit FRET [7-9]. Fab fragments, on the other hand, have a diameter of 4-8 nm [7-9]. Two fluorophores on a single Fab fragment molecule have a higher possibility to exhibit FRET with a FRET efficiency between 20-70% ( $R_0 \sim 5$  nm).

We therefore used the same labeling approaches as above but with Fab fragments as opposed to IgG (Fig. 5). Indeed, as suggested by the above line of thought, emission spectra of dual-labeled Fab fragments showed evidence of FRET between the fluorophores. The CF405M-CF488A labeled Fab fragments show an emission peak at  $\sim 450$  nm, as well as a peak at  $\sim 515$  nm as a result of the 390 nm excitation. However, this time, the



**Figure 4 | Size and FRET efficiency of IgG compared to Fab fragments. (A)** Frontal view of a full size IgG molecule for size comparison with a Fab fragment. **(B)** A plot of the FRET efficiency as a function of the distance ( $r$ ) between a donor fluorophore (green) and an acceptor fluorophore (red) with an  $R_0$  of 5 nm.



**Figure 5 | Labeling normal rabbit Fab fragments with CF405M, CF488A, CF568 and CF640R Mix-n-Stain kits.** All spectra are measured in triplicate. **(A)** CF405M-, CF488A-, and CF405M-CF488A-labeled Fab fragments at their optimal excitation wavelengths with CF405M-CF488A-labeled Fab exhibiting FRET. **(B)** CF568-, CF640R-, and CF568-CF640R-labeled Fab fragments at their optimal excitation wavelengths with CF568-CF640R-labeled Fab exhibiting FRET. Note: The y-axis here is integral normalized to make FRET (or lack thereof) apparent by comparing spectra to one another.

magnitude of the 450 nm emission peak (integral normalized intensity) is decreased compared to the pure CF405M-labeled Fab fragment; this emission intensity shows up around the 515 nm emission peak. This is a clear indicator of FRET. The CF568-CF640R labeled Fab fragments also show two emission peaks, in a similar manner that clearly indicates FRET.

## CONCLUSIONS

We demonstrated how to use Biotium Mix-n-Stain kits to multiply label Fab fragments with fluorophores that exhibit FRET. We also showed that the same protocol does not work with full IgG molecules, presumably due to their larger size. These results might lead to increased multiplexing of fluorescent antibody measurements.

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