Sapphire FL's Laser and Filter Flexibility Allows Imaging of Dyes with Large Stokes Shifts

Introduction

A wide variety of biological imaging applications take advantage of fluorescent dyes. Some dyes specifically accumulate in subcellular structures, such as mitochondria or lysosomes, allowing subcellular morphology to be studied using fluorescence microscopy. When attached to antibodies targeting specific molecules, fluorescent dyes allow imaging of target molecules in many ways. For example, fluorescently labeled target molecules are detected in tissue sections by microscopy, in cell preparations by fluorescence-activated cell sorting, or in cell lysates using fluorescent Western blots.

The utility of a fluorescent dye for use as a probe depends on several factors. The fluorescence must be strong enough to generate high signal-to-noise ratios. The signal-to-noise ratio of a target signal is affected by autofluorescence, and it is important to choose probes whose excitation and emission wavelengths avoid autofluorescence in whatever medium is being studied, such as fluid, plastic, glass, membrane, etc. Ideally, the excitation and emission spectra must be sufficiently separated so the excitation light does not bleed over into the detection channels where the emitted light is detected. This distance between the excitation and emission maxima is called the Stokes shift. As shown in Table 1, the Stokes shifts of many popular fluorescent dyes are between 10 and 40 nm. Significant work has gone into developing dyes with larger Stokes shifts for biological applications. Stokes shifts greater than 80 nm can minimize crosstalk between the excitation light and detection of emitted fluorescence during imaging, leading to better signal-to-noise ratios and better imaging.[1,2]

Most fluorescence imaging instruments have fixed channels for detecting specific, commonly used dyes. However, having fixed channels limits the development and adoption of new fluorophores that do not conform to the standard excitation/emission pairings, like dyes with very large Stokes shifts.

The Sapphire FL Biomolecular Imager provides the flexibility needed to image dyes with large Stokes shifts. Its modular excitation lasers and emission filters can be easily mixed and matched to achieve non-standard pairings compatible with specialty dyes.

This application note demonstrates the use of non-standard pairings of lasers and emission filters on the Sapphire FL to image Western blots detected with antibodies labeled with MegaStokes dyes (Table 2).

Table 1. Excitation and emission spectra of some common dyes[3]

Dye	Excitation Max (nm)	Emission Max (nm)	Stoke Shift (nm)
FITC	490	525	35
Cy2	492	508	16
Cy3	554	568	24
Cy5	649	667	18
TAMRA	550	575	25

Table 2. Excitation and emission spectra of dyes used in this application note[4]

Dye	Excitation Max (nm)	Emission Max (nm)	Stoke Shift (nm)
DY-376XL	387	549	162
DY-481XL	522	658	136

Methods

Antibody labeling and purification

Donkey anti-rabbit IgG was labeled with DY-376XL (Dyomics, product # 376XL-01) or DY481-XL (Dyomics, product # 481XL-01). Dyes were diluted with DMSO to a final concentration of 0.42 mM. Labeling reactions were prepared by combining 120 µl dye, 231 µl 1X PBS, and 250 µl 0.5 M HEPBS. The reactions incubated at room temperature for 30 minutes and were then added to a desalting column (Econo-Pac® 10DG, Bio-Rad, product # 732-2010) equilibrated with 2X PBS. The columns were washed with 1.63 mL 2X PBS, and labeled antibody was eluted with 4 mL 2X PBS. The labeled antibodies were concentrated using centrifugation in Amicon® Ultra-4 (10k) filter units (Millipore, product # UFC801096).

Western blotting

Polyacrylamide gels were run with lanes loaded with molecular weight marker (EZ sRun™, Fisher, product # BP3603-500) and a 2-fold serial dilution of HeLa cell lysate covering the range from 10 µg to 0.01 µg of total protein per lane. Protein was transferred to low fluorescence PVDF membranes (Azure, product # AC2105) using semidry transfer.

Blots were blocked with Azure Fluorescent Blot Blocking Buffer (Azure product # AC2190) and probed with a rabbit antibody directed against STAT3 (CST product # 12640S) for 60 minutes at room temperature. Blots were washed 3 times for 5 minutes each with 1X Azure Blot Washing Buffer (Azure product # AC2113) and then probed with the MegaStokes dye-labeled goat anti-rabbit antibodies for 30 minutes at room temperature. Blots were washed three times for 5 minutes each with Azure Blot Washing Buffer and then once with 1X PBS for 5 minutes and quickly with methanol to dry. Blots were imaged immediately.

Imaging

Blots were imaged with the Sapphire FL Biomolecular Imager.

The blot detected using the MegaStokes 376XL dye was imaged using the Sapphire FL 375 nm laser (Azure product # IS4023) and the Sapphire FL 534/20 emission filter (Azure product # IS4010). Intensity setting was 10, quality was set to highest, resolution was 20 microns, and focus type was set to membrane (focus height 0 mm, directly on glass scanning surface).

The blot detected using MegaStokes 481XL dye was imaged using the Sapphire FL 488 nm laser (Azure product # IS4025) and the Sapphire FL 624/40 emission filter (Azure product # IS4013). Intensity setting was 9, quality was set to highest, resolution was 20 microns, and focus type was set to membrane (focus height 0 mm, directly on glass scanning surface).

Results and Discussion

Two Western blots detected using MegaStokes dyes were imaged using the Sapphire FL Biomolecular Imager. Figure 1 shows a blot of HeLa cell lysate probed for STAT3 and detected with DY-376XL and Figure 2 shows a duplicate blot also probed for STAT3 and detected with DY-481XL. Both dyes have Stokes shifts well over 100 nm (Table 2), requiring the use of non-standard pairings of lasers and filters. The module-

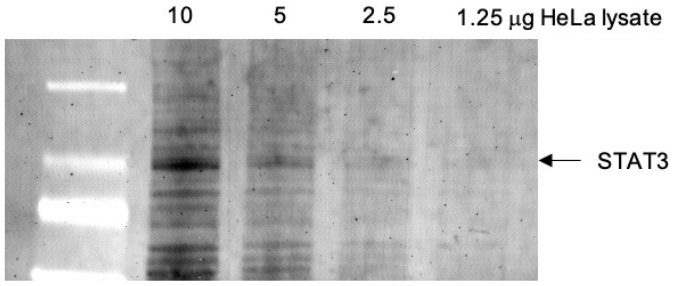

Figure 1. Western blot detecting STAT3 in serial dilutions of HeLa cell lysate. STAT3 was detected using a DY-376XL-labeled antibody and imaged on the Sapphire FL using the 375 nm laser and 534/20 emission filter.

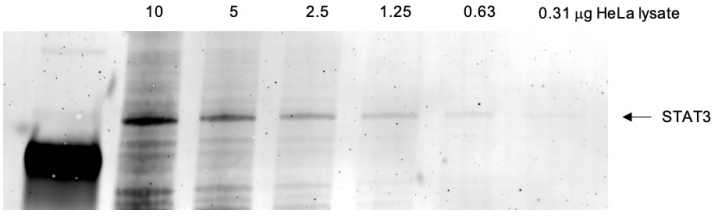

Figure 2. Western blot detecting STAT3 in serial dilutions of HeLa cell lysate. STAT3 was detected using a DY-481XL-labeled antibody and imaged on the Sapphire FL using the 488 nm laser and 624/40 emission filter.

based design of the Sapphire FL makes it possible to mix and match lasers and filters as needed, which can facilitate the imaging of almost any fluorescent dye.

As seen in Figures 1 and 2, STAT3 was detected in as little as 1.25 µg of HeLa cell lysate with the DY-376XL-labeled secondary antibody, and in as little as 0.31 µg of HeLa cell lysate with the DY-481XL-labeled antibody. This difference could be due to differences in labeling efficiency of the secondary antibodies.

The unique flexibility of the Sapphire FL makes it an ideal choice when imaging blots or tissues labeled with fluorescent dyes having large Stokes shifts. Azure offers many standalone laser diodes and standalone emission filter options that can be combined to allow detection of almost any fluorescent dye. Custom laser and emission filter options are also available. In addition to multichannel fluorescence, the Sapphire FL can carry out white light, UV, chemiluminescence, bioluminescence, and phosphor imaging. Learn more about the Sapphire FL at https://azurebiosystems.com/products/sapphirefl/.

References

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MM-0167 R2