Enhance immunodetection in neurons and other complex samples

Development and optimization of Alexa Fluor Plus secondary antibodies.

Obtaining the best possible results in fluorescence-based cell imaging requires that all reagents used in the workflow are optimized to maximize signal over background. Optimization is especially critical when working with challenging experimental systems, samples that are hard to obtain like neurons, or low-abundance cellular targets that require signal amplification. An essential yet often overlooked factor in the workflow is the selection and optimal use of the fluorescent secondary antibodies. Invitrogen™ Alexa Fluor™ Plus secondary antibodies were specifically developed to provide superior sensitivity, a high signal-to-noise (S/N) ratio, and low cross-reactivity for the detection of low-abundance proteins in critical samples and experiments. This article describes how we developed these antibodies using a high-throughput workflow that allowed us to perform complex, multivariable analyses on the different antibody candidates under a range of experimental conditions, resulting in the selection of the best possible Alexa Fluor Plus antibody conjugates.

Overview of the high-throughput workflow

Testing multiple dyes and dye conjugates across a range of samples and dilutions requires a high-throughput solution that enables reliable quantitation, reproducibility, scalability, and analysis of hundreds of individual data points. To accomplish this task, we utilized the Thermo Scientific™ CellInsight™ CX5 High-Content Screening (HCS) Platform equipped with Thermo Scientific™ HCS Studio™ Cell Analysis Software and semi-automated sample handling. Cells were seeded, fixed, and permeabilized in 96-well or 384-well plates, with washing and processing performed on a Thermo Scientific™ Multidrop™ Combi Reagent Dispenser. Multiwell replicates of cells were incubated in the presence (signal) or absence (background) of primary antibody, followed by secondary antibody incubation, with matched conditions for washing, nuclear staining, and signal acquisition across all wells.

Finding the optimal working concentration for potential Alexa Fluor Plus candidates

The first step of the testing process was focused on determining the optimal working concentration for all of the Alexa Fluor Plus secondary antibody candidates. In the example shown in Figure 1, the fluorescence intensity of goat anti–mouse IgG secondary antibodies was measured with or without primary antibody at concentrations spanning 0.1 ng/mL to >10 µg/mL (Figure 1A–C). Interestingly, background fluorescence in the secondary antibody control (samples without primary antibody) increased drastically at concentrations over 10 µg/mL (Figure 1B), resulting in suboptimal S/N ratios at these higher secondary antibody concentrations (Figure 1C). It is important to note that the optimal secondary antibody concentration can vary with the label and species specificity of the secondary antibody, as

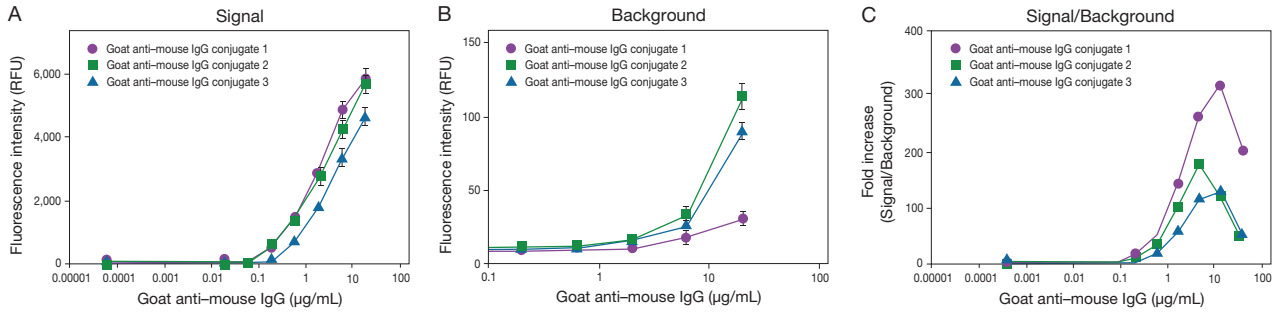

Figure 1. Titration of fluorescent secondary antibodies for optimized signal-to-noise (S/N) ratio. Fluorescent secondary antibodies—including Invitrogen™ Alexa Fluor™ Plus development candidates—were titrated to determine the optimal concentration range for maximal S/N ratio. Fluorescence intensities are shown as relative fluorescence units (RFU) using **(A)** secondary antibody in the presence of a corresponding primary antibody to determine signal or **(B)** secondary antibody in the absence of a primary antibody, to control for nonspecific background noise due to the secondary antibody. **(C)** The S/N values for data in panels A and B are shown. The optimal S/N ratio for all secondary antibody candidates is obtained using 1–10 µg/mL and declines with higher concentrations of secondary antibody. Data were acquired on a Thermo Scientific™ CellInsight™ CX5 High-Content Screening Platform equipped with Thermo Scientific™ HCS Studio™ Cell Analysis Software.

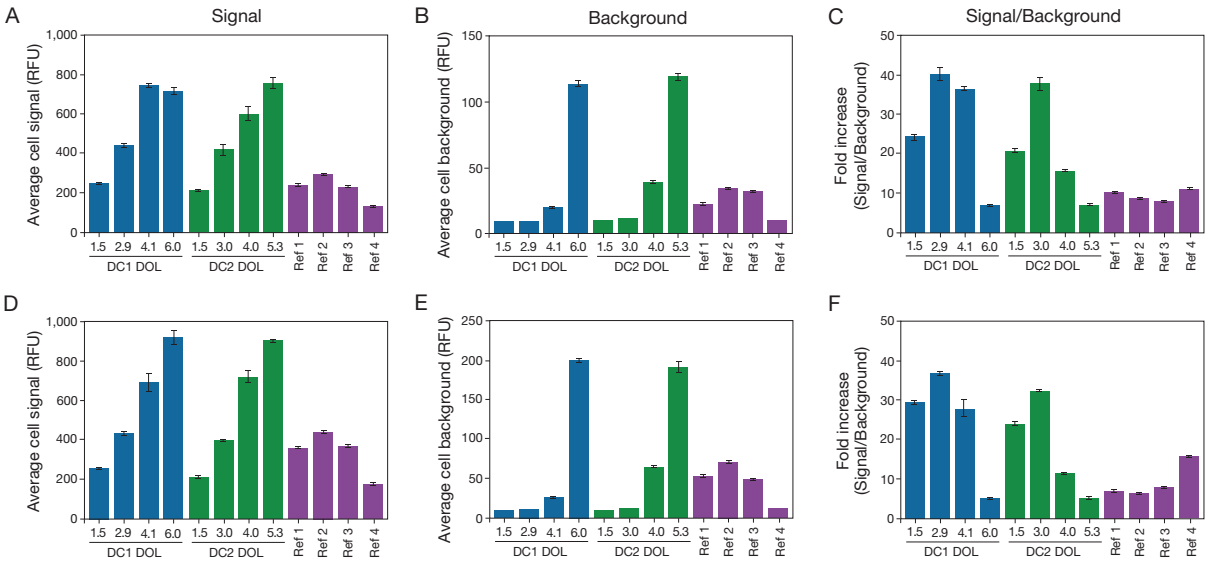

Figure 2. Using a guard banding approach to determine the optimal degree of labeling (DOL) of Alexa Fluor Plus secondary antibodies. DOL guard banding of two Invitrogen™ Alexa Fluor™ Plus development candidates (for the Alexa Fluor Plus 594 goat anti–mouse IgG secondary antibody) and benchmark secondary antibodies in **(A–C)** cytoplasmic and **(D–E)** nuclear cell staining applications. **(A, D)** Signal and **(B, E)** background are shown as average cell intensities (in RFU); **(C, F)** signal-to-noise (S/N) ratios are expressed as fold increase of the signal over background. All determinations were generated with RFU data collected from 200 cells per well, n = 3 wells per condition tested. Data were acquired on a Thermo Scientific™ CellInsight™ CX5 High-Content Screening Platform equipped with Thermo Scientific™ HCS Studio™ Cell Analysis Software.

well as with the primary antibody. Therefore, we always recommend that the secondary antibody be titrated for each primary antibody used in an imaging experiment to help ensure that optimal S/N ratios are obtained. Omitting this titration could result in a decreased range of detection and a lower S/N ratio, which can translate into loss of signal specificity or increased background.

Screening Alexa Fluor Plus candidates with different DOLs for the optimal S/N ratios

To determine the optimal degree of labeling (DOL) of Alexa Fluor Plus conjugates in development, we used highly cross-adsorbed IgGs that were labeled and purified with a range of DOLs using an approach called guard banding, which defines the acceptable offset from a given specification (in this case, the DOL that produces the optimal S/N). These conjugates were then used for a series of determinations of signal intensity and background fluorescence at various concentrations in cells that had been incubated with or without primary antibody.

Figure 2 shows an example of DOL guard banding of two development candidates (DC1 and DC2) that were tested in parallel at 2 µg/mL to determine 1) optimal DOL range and 2) which candidate to use for the Alexa Fluor Plus 594 goat anti–mouse IgG secondary antibody, based on S/N performance. In this example, the secondary antibodies were used to detect an anti-tubulin primary antibody in the cytoplasm of A549 cells (Figure 2A–C). A no–primary antibody control was used to detect background in the same area of the cytoplasm. DC1 and DC2 were prepared with DOLs between 1.5 and 6.2 and benchmarked to spectrally similar red-fluorescent goat anti–mouse IgG secondary antibodies from Thermo Fisher Scientific and other suppliers at matched concentrations (Figure 2, purple bars). As expected, as the average number of fluorophores per antibody molecule increased, the signal intensity (measured in relative fluorescence or RFU) for DC1 (blue bars) and DC2 (green bars) also increased, from an average of 200 RFU for a DOL of 1–2 to over 700 RFU for a DOL of 4–6 (Figure 2A). However, increasing the DOL can also lead to an increase in background, as shown in this example for the samples with high DOL values of 5–6 (Figure 2B). Ultimately, the S/N ratio was used as the critical performance indicator when evaluating the development candidates (Figure 2C). Both development candidates showed higher S/N ratios over benchmark secondary antibodies; however, DC1 showed a higher S/N ratio over a broader range of DOLs (Figure 2C).

To confirm these results using a nuclear target, we performed the same guard banding approach by probing A549 cells for etoposide-induced DNA damage and subsequent phosphorylation of the histone H2AX (pH2AX) using an anti-pH2AX primary antibody. Next we →

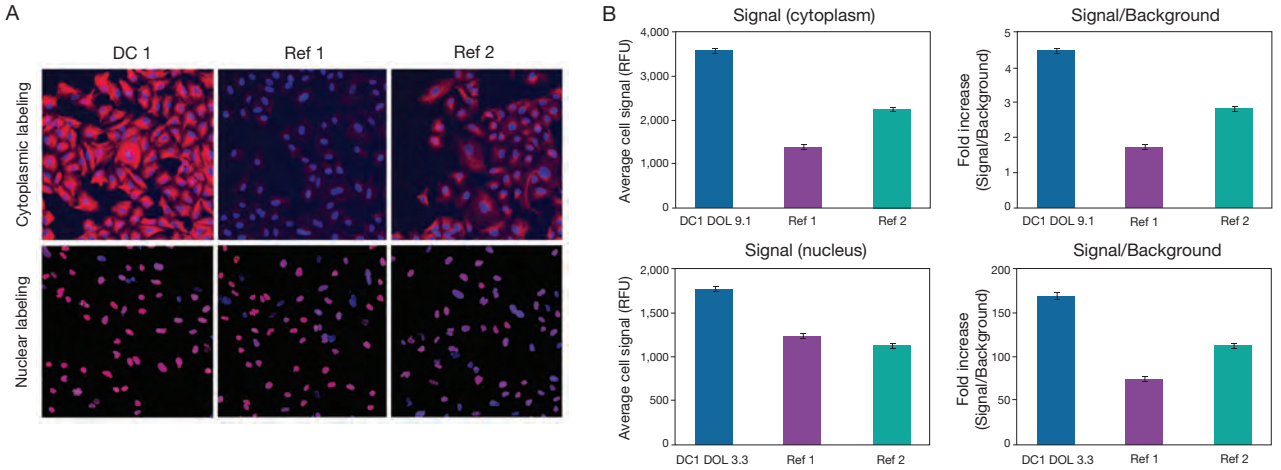

Figure 3. Comparison of cytoplasmic and nuclear target signal-to-noise (S/N) ratios using Alexa Fluor Plus antibody conjugates. (A) Top row: Representative pseudocolored images show the differences in signal intensity and S/N ratios after staining with Invitrogen™ Alexa Fluor™ Plus 555 donkey anti–mouse IgG antibody (Cat. No. A32773) and benchmark secondary antibody (Ref 1 and Ref 2, above) for the detection of tubulin in the cytoplasm (red) of A549 cells, counterstained with DAPI (blue). Bottom row: Alexa Fluor Plus 594 goat anti–rabbit IgG antibody (Cat. No. A32740) and benchmark secondary antibody (Ref 1 and Ref 2, above) were used for the detection of pH2AX in the nuclei of etoposide-treated A549 cells (purple) counterstained with DAPI (blue). **(B)** Quantitation of average signal intensity and S/N values are also shown.

measured the average signal intensity and background fluorescence (with and without primary antibody) in cell nuclei (Figure 2D–E). Cells were labeled with the same two secondary antibody candidates using the same DOL range as in Figure 2A–C, and the S/N ratios were analyzed. As shown in Figure 2D–E, the results were very consistent between cytoplasmic and nuclear staining, confirming that both development candidates showed higher S/N ratios over the benchmark secondary antibodies and that DC1 showed higher S/N ratios over a broader range of DOLs for this nuclear target. Based on these results, DC1 was selected for the new Alexa Fluor Plus 594 goat anti–mouse IgG secondary antibody with a DOL range of 2–4.

Table 1 provides an overview of two of the six species-specific Alexa Fluor Plus secondary antibodies (donkey anti–mouse IgG and donkey anti–rabbit IgG), with DOL ranges, excitation/emission maxima, and tested applications; Figure 3 shows representative pseudocolored images. It is important to note that, depending on the abundance of the target, performance of the primary antibody, and complexity of the cell system, Alexa Fluor Plus secondary antibodies enable a significantly more robust detection of target proteins in most cases, primarily by boosting signal intensity, decreasing background levels, or both.

Testing Alexa Fluor Plus antibodies in neuronal systems

Alexa Fluor Plus secondary antibodies were used in a variety of cell systems. Two recent examples are shown in Figures 4 and 5 and in several published articles [1-4]. In Figure 4, Alexa Fluor Plus 488 donkey anti–mouse IgG and Alexa Fluor Plus 555 donkey anti–rabbit IgG secondary antibodies (Figure 4A) were used for the detection of neuronal markers HuC/D and MAP2 in rat cortical neurons (Figure 4A), and compared with results obtained with the benchmark secondary antibodies (Figure 4B). This comparison demonstrates that the Alexa Fluor Plus secondary antibodies, optimized during development using the CellInsight CX5 HCS Platform, deliver the expected superior performance over benchmark secondary antibodies, providing higher S/N

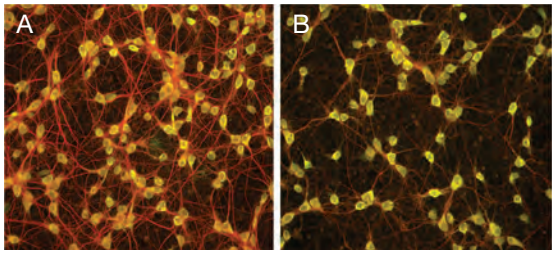

Figure 4. Enhanced detection of neuronal detail using Alexa Fluor Plus antibody conjugates. Invitrogen™ Alexa Fluor™ Plus 555 donkey anti–rabbit IgG (Cat. No. A32794) and Invitrogen™ Alexa Fluor™ Plus 488 donkey anti–mouse IgG (Cat. No. A32766) secondary antibodies were used to detect anti-MAP2 (red) and anti-HuC/D (green/yellow, as a result of overlay with red MAP2 labeling) primary antibodies in rat cortical neurons. Contrast-matched images show comparison of staining with **(A)** Alexa Fluor Plus vs. **(B)** benchmark secondary antibodies. Data were acquired on a Thermo Scientific™ CellInsight™ CX5 High-Content Screening Platform equipped with Thermo Scientific™ HCS Studio™ Cell Analysis Software.

ratios and greater image detail in fluorescence microscopy applications. In Figure 5, Alexa Fluor Plus 555 donkey anti–mouse IgG and Alexa Fluor Plus 647 donkey anti–rabbit IgG secondary antibodies used in a cardiovascular lateral flow experimental system for detection of membrane-associated proteins improved the sensitivity of target detection over that obtained using benchmark secondary antibodies (Figure 5B) in the same system under matched conditions.

Use Alexa Fluor Plus secondary antibodies for your experiments

For the detection of low-abundance targets in immunofluorescence imaging, there are multiple options available to researchers, each with its unique set of pros and cons. Depending on the experimental system or the level of signal amplification needed, the use of biotinylated secondary antibodies in conjunction with dye-labeled streptavidin conjugates or tyramide-based signal amplification may be considered. However, due to its simplicity and consistency, the most common signal amplification technique is the use of fluorescent secondary antibodies.

Alexa Fluor Plus secondary antibodies were developed to deliver more sensitive fluorescence detection of primary antibodies with greater S/N ratios, enabling more robust detection of low-abundance targets in hard-to-obtain samples and critical experiments. The Alexa Fluor Plus secondary antibodies, available in a wide range of fluorescent colors and species specificity (Table 2), have been widely referenced in scientific publications and used in a great variety of cell model systems and applications. To learn more and find a complete list of available Alexa Fluor Plus secondary antibodies, go to thermofisher.com/alexafluorplus. ■

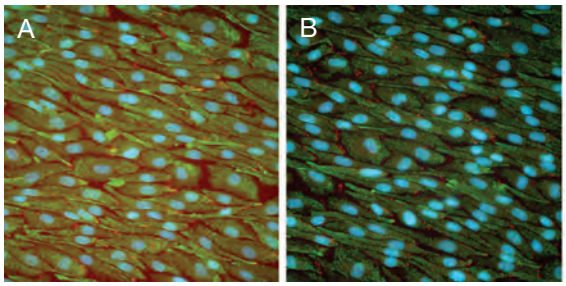

Figure 5. Improved sensitivity and specific signal brightness with Alexa Fluor Plus antibody conjugates. A flow-aligned human aortic endothelial cell (HAEC) monolayer stained for plasma membrane–associated proteins using **(A)** Invitrogen™ Alexa Fluor™ Plus 555 donkey anti–mouse IgG (green, Cat. No. A32773) and Invitrogen™ Alexa Fluor™ Plus 647 donkey anti–rabbit IgG (red, Cat. No. A32795) or **(B)** traditional Alexa Fluor™ secondary antibodies under matched conditions. Data used with permission from Julia J. Mack and Julianne W. Ashby, Department of Medicine/Cardiology, University of California Los Angeles.

References

1. Maximov V, Chen Z, Wei Y et al. (2019) *Nat Commun* 10:2410.
2. Kleene SJ, Siroky BJ, Landero-Figueroa JA et al. (2019) *PLoS One* 14:e0214053.
3. Harris C, Cloutier M, Trotter M et al. (2019) *eLife* 8:e44258.
4. Xia L, Li S, Liu Y et al. (2019) *Oncol Lett* 17:2969–2975.

Table 1. DOL ranges for Alexa Fluor Plus donkey anti–mouse IgG and donkey anti–rabbit IgG secondary antibodies and their applications.

	Conjugate	DOL range	Ex/Em max (nm)	Applications (tested, published)	Cat. No.
Donkey anti–mouse	Alexa Fluor Plus 488	6–10	490/525	IF/ICC [1,2], WB	A32766
	Alexa Fluor Plus 555	8–11	555/580	IF/ICC [3], WB	A32773
	Alexa Fluor Plus 594	2–4	590/617	IF/ICC	A32744
	Alexa Fluor Plus 647	5–8	650/665	IF/ICC, WB	A32787
	Alexa Fluor Plus 680	5–8	679/702	IF/ICC, WB	A32788
	Alexa Fluor Plus 800	2–5	777/794	IF/ICC, WB	A32789
Donkey anti–rabbit	Alexa Fluor Plus 488	5–8	490/525	IF/ICC, WB	A32790
	Alexa Fluor Plus 555	7–10	555/580	IF/ICC, WB	A32794
	Alexa Fluor Plus 594	2–3.5	590/617	IF/ICC [2]	A32754
	Alexa Fluor Plus 647	4–7	650/665	IF/ICC [4], WB	A32795
	Alexa Fluor Plus 680	4–6	679/702	IF/ICC, WB	A32802
	Alexa Fluor Plus 800	2–4	777/794	IF/ICC, WB	A32808

Table 2. Alexa Fluor Plus secondary antibody catalog numbers, by species specificity and fluorescent color.

Secondary antibody	Alexa Fluor Plus 488	Alexa Fluor Plus 555	Alexa Fluor Plus 594	Alexa Fluor Plus 647	Alexa Fluor Plus 680	Alexa Fluor Plus 800
Goat anti–rabbit IgG	A32731	A32732	A32740	A32733	A32734	A32735
Goat anti–mouse IgG	A32723	A32727	A32742	A32728	A32729	A32730
Goat anti–chicken IgY	A32931	A32932	A32759	A32933	A32934	A32935
Donkey anti–goat IgG	A32814	A32816	A32758	A32849	A32860	A32930
Donkey anti–rabbit IgG	A32790	A32794	A32754	A32795	A32802	A32808
Donkey anti–mouse IgG	A32766	A32773	A32744	A32787	A32788	A32789