Product Information

Basic Protocols for Fluorescence Detection using Secondary Antibodies

The following are general protocols for immunofluorescence staining with CF® Dye labeled antibodies for the detection of proteins by microscopy or western blot. Please note that there are many variations of antibody detection methods. These protocols are intended as general guidelines and should be optimized for best results. Additional protocols, for example for staining cells for flow cytometry, can be found in our Protocols Library. To view our full selection of secondary antibodies, please visit our Secondary Antibody Product Listings.

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Immunofluorescence Staining for Microscopy

This protocol is for staining fixed and permeabilized cells. Staining of tissue sections may require antigen retrieval or autofluorescence quenching. For more information, see Tech Tip: Considerations for Immunofluorescence Staining.

Materials

- PBS or HBSS (buffer with Ca^{2+}/Mg^{2+} is usually recommended for maintaining adhesion and morphology of adherent cells)
- Fixative: 4% paraformaldehyde in PBS (at room temperature or 4°C), or methanol (pre-chilled to -20°C)
- Blocking/permeabilization buffer: TrueBlack® IF Background Suppressor System, or buffer of your choice (e.g., PBS + 2% fish gelatin + 0.1% Triton® X-100 + 0.02% sodium azide)
- Primary antibody
- CF® Dye Secondary Antibody
- EverBrite™ Mounting Medium
- Nail polish or CoverGrip™ Coverslip Sealant (for wet-mounted coverslips)

Workflow overview

- Fix (≤ 20 minutes) (optional stopping point)
- Block/permeabilize (30 minutes) (optional stopping point)
- Primary antibody incubation (2 hours or overnight)
- Washes (20-30 minutes)
- Secondary antibody conjugate incubation (30 minutes to 2 hours)
- Washes (20-30 minutes)
- Mount (optional stopping point)
- Image slide

General considerations for immunofluorescence staining

- In our experience, cells can be stored in PBS after fixation for several weeks. We recommend using PBS with 0.02% sodium azide as a preservative. Keep samples well-sealed or in a humidified box to avoid evaporation of buffer.
- Different fixation methods may be optimal for your target of interest. If the optimal fixation conditions are unknown, it may be necessary to test different fixation methods for a specific target.
- Other blocking reagents, such as BSA, may also be used instead of fish gelatin. When using some highly negative charged fluorescent dyes, specialized blocking buffers, such as our TrueBlack® IF Background Suppressor System (Cat. No. 23012), may help reduce background.
- Conjugates of blue fluorescent dyes like CF®405S are not as bright as other colors and are not recommended for detecting low-abundance targets. Blue dyes are also challenging to use in tissue specimens, which have high autofluorescence in blue wavelengths.

Protocol

1. Remove the culture medium from adherent cells, then wash the cells twice with PBS or HBSS to remove any remaining traces of medium. Use the same volume for washes as you would for cell culture medium (we use 100 uL per well of a 96-well plate). For some cell types, buffer with Ca^{2+}/Mg^{2+} may be necessary to prevent cell rounding and detachment. Prior to fixation, we prefer to use HBSS with Ca^{2+}/Mg^{2+} for adherent cells.
2. Fix cells with 4% paraformaldehyde in PBS for 20 minutes at room temperature or 4°C. Alternatively, fix cells in pre-chilled methanol at -20°C for 5-10 minutes.
3. Rinse cells three times with PBS to remove traces of fixative.
4. Block and permeabilize cells with blocking/permeabilization buffer of your choice.
5. Dilute the primary antibody in fresh blocking/permeabilization buffer at the concentration recommended by the antibody supplier.

   Note: You may need to perform a titration experiment to determine the optimal concentration of your primary antibody.

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6. Add enough diluted antibody solution to cover the cells completely. We usually use 50-100 uL per well of a 96-well plate.

   Note: For cells on coverslips, add 50-100 uL of diluted antibody solution and overlay with a piece of Parafilm® to spread the solution evenly over the specimen, making sure there are no bubbles. Keep the samples in a humidified chamber to avoid evaporation.

7. Incubate 1-2 hours at room temperature or overnight at 4°C (in our experience, 4°C overnight gives the best results).

8. Rinse cells twice with PBS, then wash 3 x 5 minutes with PBS. Alternatively, rinse cells twice with PBS, incubate in PBS for 30 minutes, then rinse with PBS. Cells can be left in PBS for longer times without negatively affecting staining.

9. Dilute the secondary antibody in blocking/permeabilization buffer at 1-2 ug/mL. Cover cells with secondary antibody solution as in step 6 and incubate for 30 minutes to 2 hours at room temperature, protected from light. The appropriate antibody dilution should be determined empirically. Generally, IgG conjugates are used in the range of 1-10 ug/mL. Additionally, nuclear stains or other counterstains, such as phalloidin conjugates, may be co-incubated with the secondary antibody.

10. Wash cells as in step 8.

11. Mount the samples in fluorescence antifade mounting media, such as EverBrite™ Mounting Medium. For chambered coverglass or multi-well coverglass plates, remove all traces of buffer and add enough mounting medium to completely cover the cells.

    Notes:

    a. Mounting medium with nuclear staining dye can be used, such as EverBrite™ Mounting Medium with DAPI (Cat. No. 23022) or EverBrite™ Hardset with NucSpot® 640 (Cat. No. 23016).

    b. For coverslips, wet-set or hard-set mounting medium may be used. Follow mounting medium instructions for mounting coverslips. If wet-set mounting medium is used, the edges of the coverslip must be sealed with nail polish or CoverGrip™ Coverslip Sealant (recommended) before imaging.

12. Store your samples at 4°C and protected from light until ready to image. Usually, antibody-stained samples can be stored in mounting medium at 4°C for six months or longer.

Fluorescent Western Blotting

Materials

- VersaBlot™ Total Protein Normalization Kit (optional)
- TrueBlack® WB Blocking Buffer Kit (or other preferred blocking buffer)
- PBS or TBS with 0.1% Tween®-20
- Primary Antibody
- CF® Dye Secondary Antibody

Workflow overview

- Optional: Total protein prestaining
- SDS-PAGE and protein transfer (~2 hours) (optional stopping point)
- Optional: Confirm protein transfer
- Blocking (30-60 minutes)
- Primary antibody incubation (2 hours or overnight)
- Washes (30-60 minutes)
- Secondary antibody conjugate incubation (30 minutes to 2 hours)
- Washes (30-60 minutes)
- Dry membrane (optional stopping point)
- Image membrane

General considerations for fluorescent western detection

- Far-red or near-infrared dyes are optimal for fluorescent western, because background is lower in these wavelengths. Visible fluorescent dyes can be used, but generally will have lower signal-to-noise ratio due to higher autofluorescence of proteins and blotting membranes in the visible spectrum.
- Multiplex fluorescence western detection requires an imaging system capable of detecting multiple fluorescent conjugates. For best results, use a gel imager or scanner specifically designed for imaging fluorescent blots.
- Reducing or non-reducing SDS-PAGE may be optimal for detecting different proteins. We recommend checking the literature, or comparing reduced and non-reduced samples to determine optimal conditions.
- Optimal protein loading amount varies depending on the detection method and target expression level, but normally ranges between 1-10 ug/lane.
- As a protein marker, we recommend using the Peacock™ Plus Prestained Protein Marker (Cat. No. 21531), which has bands that range from 8 kDa to 245 kDa. The bands fluoresce in the 700 channel and are visibly colored blue and red, so you can monitor your gel electrophoresis and transfer. We recommend using 1.5-3 uL of ladder per lane for fluorescence detection.
- Blue tracking dyes in SDS-PAGE loading buffer can fluoresce in the far-red/near-infrared spectra. Loading buffer with an orange tracking dye is recommended for fluorescent western detection. Biotium offers a 4X Protein Loading Buffer with Orange Tracking Dye (Cat. No. 40136).

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- We recommend using low-fluorescence PVDF for fluorescent western blot detection. Nitrocellulose membranes may also be used and, in our experience, have shown similar background fluorescence to low-fluorescence PVDF.
- Ponceau S Solution is not recommended for near-IR western blots due to poor sensitivity, especially on PVDF. For total protein staining, we recommend VersaBlot™ Total Protein Normalization Kits (Cat. No. 33025, 33026) due to their exceptional linearity, ease of use, and downstream reversibility for multi-color analysis.
- 9 cm^2 petri dishes hold 5-10 mL and are convenient for washing and incubating mini-blots. Alternatively, commercially available black blotting boxes for fluorescent westerns come in a variety of sizes for blots of membrane strips.
- Either PBS or TBS can be used for fluorescent western detection with similar results.
- TrueBlack® WB Blocking Buffer (Cat. No. 23013), or other commercially available blocking buffers developed specifically for fluorescent western detection, can give lower background than other blocking agents. However, BSA, non-fat dry milk, or fish gelatin can be used for western blot blocking and antibody dilution buffers. These blocking agents are usually used at 1-5% in PBS (or TBS) + 0.1% Tween®-20.
- It may be desirable to minimize the volume of antibody solutions used for blotting by using sealable bags or small containers. Enough solution should be used to freely move across the blot without trapping bubbles.
- For blocking and wash steps, always use plenty of liquid to cover the blot. Use 5-10 mL buffer for a mini-blot. The blot should move freely in the buffer.
- If you want to use antibodies from the same organism for fluorescent western detection, you may use fluorescently labeled primary antibodies.

Protocol

1. Optional: To fluorescently label total protein in your samples for transfer confirmation and western normalization, use a total protein prestaining kit, such as our VersaBlot™ Total Protein Normalization Kit (Cat. No. 33025, 33026), according to the kit protocol.
2. Perform SDS-PAGE and western transfer using standard protocols. See general considerations for fluorescent western detection for protein loading recommendations.

   Note: After transfer, membranes can be rinsed in water, dried, and stored between sheets of filter paper at room temperature for months or longer.
3. Optional: Confirm protein transfer by imaging total protein prestain, if used.
4. If using PVDF membranes, re-wet the membrane in methanol, then rinse in water. For nitrocellulose membranes, proceed to step 5.
5. Place the membrane in a clean dish containing blocking buffer of your choice. Use enough buffer to completely cover the blot and allow it to move freely in the dish.
6. Block the membrane for 30-60 minutes at room temperature with gentle rocking.
7. Dilute the primary antibody to the recommended concentration in fresh blocking buffer. Pour off the blocking buffer and add enough diluted antibody solution to allow the membrane to move freely with no stationary bubbles or dry spots.
8. Incubate the membrane with gentle rocking for 1-2 hours at room temperature or overnight at 4°C. If using fluorescently labeled primary antibodies, protect from light.
9. Rinse the membrane three times with PBS or TBS with 0.1% Tween®-20, then wash 3-5 times for 5-10 minutes each with rocking. Use a generous amount of wash buffer so membranes move freely during washes.
10. Dilute the secondary antibody in fresh blocking buffer in the range of 50-100 ng/mL. Add to blot as in step 7.
11. Incubate the membrane with gentle rocking for 1-2 hours at room temperature or overnight at 4°C. Protect from light.
12. Wash the membrane as in step 9.
13. Rinse the membrane once in buffer without detergent and dry before imaging using a compatible fluorescence imaging system.

    Notes:

    a. Dried blots can be stored between sheets of filter paper at room temperature in the dark and re-scanned after months or even years.

    b. Keep blots wet at all times and store in buffer if they are to be stripped and probed with additional antibodies.

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Troubleshooting Tips for Antibody Staining

Problem	Potential Causes/Diagnosis	Potential Solutions
High background or non-specific staining	Cross-reactivity of secondary antibody with other antibodies or proteins in sample Perform staining controls with secondary antibody alone to determine whether the secondary antibody is binding the sample directly. For multiple staining experiments, stain with each primary and secondary combination separately to detect unexpected antibody cross-reactivity.	• For indirect staining (primary + secondary antibody) with multiple primary antibodies, use secondary antibodies that are highly cross-adsorbed to prevent cross-reactivity. • When staining rat tissue with anti-mouse antibodies, use secondary antibodies that are highly cross-adsorbed against rat. • Staining of mouse tissues with anti-mouse antibodies (known as mouse-on-mouse staining) may require special protocols to block binding of endogenous antibodies in the tissue. • Highly charged fluorescent dyes, including CF®405S, Alexa Fluor® 647, or Cy®5.5 can contribute to non-specific binding of conjugates.
	Fluorescence cross-talk between channels For multi-color experiments, perform controls with each stain alone, and image in all channels to determine whether there is fluorescence cross-talk or bleed-through of dye fluorescence between channels.	• Choose dyes that are spectrally well-separated for multi-color imaging. Biotium's Spectra Viewer can be useful for this purpose. • Multi-color flow cytometry analysis may require fluorescence compensation. See your cytometer user manual for information. • Confocal microscopy imaging settings can be optimized to minimize cross-talk by limiting cross-excitation during scanning, or by changing the emission cut-off for different dyes. • To minimize DAPI fluorescence in the green channel, reduce the concentration of DAPI, or optimize confocal imaging settings to prevent cross-talk. Nuclear counterstains for other channels, such as RedDot™2 or NucSpot® Nuclear Stains, can be used to avoid this problem.
	If both signal and background are high, antibody concentration may be too high.	Perform a titration of antibody concentration to find the optimal concentration. The optimal concentration for primary antibodies can vary widely. See the application protocols for recommended starting concentrations for titration.
	Cell or tissue autofluorescence causing background during microscopy **Note:** Autofluorescence is a major and nearly universal source of background in tissue sections, and also is present in some primary cells and pigmented cell types. Include an unstained control to determine the level of autofluorescence in your sample.	• Cellular autofluorescence is high in blue wavelengths, so avoid using blue fluorescent conjugates for low expressing targets. Autofluorescence may also occur in other channels in certain organisms, and we recommend always including an appropriate control. Consult the relevant literature for more information. • Use TrueBlack® Lipofuscin Autofluorescence Quencher (Cat. No. 23007, 23011) to quench tissue autofluorescence. • Amplify your specific signal over background by using indirect immunofluorescence (primary + secondary antibody) or tyramide signal amplification.
	Blotting membrane autofluorescence Scan an unused blotting membrane next to your western blot to determine the level of autofluorescence in your sample.	Use low fluorescence PVDF for fluorescent western detection. In our experience, nitrocellulose and low fluorescence PVDF membranes show similar background fluorescence, but PVDF can give higher sensitivity, possibly due to higher protein binding.
	Suboptimal western blot blocking	Test different blocking agents to find the optimal conditions, or try a blocking buffer specifically designed for fluorescent westerns, like the TrueBlack® WB Blocking Buffer Kit (Cat. No. 23013).
	Insufficient washing of western blots	Increasing the number of washes can improve background for western blots. Use a generous volume of wash buffer with rocking so blots move freely during washing.

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Troubleshooting Tips for Antibody Staining (continued)

Problem	Potential Causes/Diagnosis	Potential Solutions
No staining or low signal	Antibody conjugate not validated for application	• Check that the antibody conjugate is recommended for your application. • Validate antibody with positive control cell line or tissue that expresses the target. • Check that the species reactivity of the antibody is compatible with your sample.
	Target protein not expressed	Check literature or Human Protein Atlas to confirm the protein is expressed in your sample.
	Antibody concentration too low	Perform a titration of antibody concentration to find the optimal concentration. The optimal concentration for primary antibodies can vary widely; concentrations for initial testing usually start around 1 ug/mL or higher.
	Secondary antibodies not compatible with serum proteins used for blocking	If using anti-goat or anti-bovine secondary antibodies, avoid blocking buffers with milk, goat serum, or bovine serum albumin.
	Intracellular target or epitope not accessible for surface staining for microscopy Check that the antibody epitope is in an extracellular domain of the target protein. Perform intracellular staining to determine if target is localized inside the cell.	• Use an antibody raised against an extracellular domain of the protein, or that is validated for surface staining. • Perform intracellular staining for intracellular targets.
	Fluorescence photobleaching during microscopy	Use antifade mounting medium. Some fluorescent dyes are more photostable than others, choose photostable dyes like rhodamine-based CF® Dyes for microscopy applications.
	Imaging settings not compatible with dyes	Check that you are using the correct excitation/emission settings for the dyes. Note that far-red conjugates are not visible to the human eye, and must be imaged using a CCD camera or confocal microscope.

Products for Immunofluorescence and Western Blotting

Product	Cat. No.	Features
Mix-n-Stain™ CF® Dye Antibody Labeling Kits	92230-92584	• Optimized for stable labeling of IgG antibodies • Choice of over 30 CF® Dye colors, biotin, or a variety of classic labels • Labels IgG in as little as 15 minutes without a purification step • Labeling tolerates many common buffer components including BSA and ascites
TrueBlack® IF Background Suppressor System (Permeabilizing)	23012	• Suppress background from non-specific antibody binding and charged fluorescent dyes • More efficient than Image-iT® FX; block & permeabilize in just 10 minutes • Complete system for blocking, permeabilizing, and antibody dilution • For staining of cells or tissue sections
TrueBlack® WB Blocking Buffer Kit	23013	• Blocks non-specific background fluorescence over the entire membrane • Works as well or better than LI-COR's Odyssey® Blocking Buffer and at a lower cost • Compatible with PVDF and nitrocellulose membranes • Can be used with fluorophores spanning the visible and NIR spectra
TrueBlack® Lipofuscin Autofluorescence Quencher	23007, 23011	• Eliminates lipofuscin autofluorescence with less background than Sudan Black B • Reduces background from other sources like red blood cells and extracellular matrix • Can be used before or after IF staining
TrueBlack® Plus Lipofuscin Autofluorescence Quencher	23014	
EverBrite™ Mounting Medium	23001-23002	• Excellent protection from photobleaching for a wide range of dyes, including cyanine • Available in wet-set or hardset formulations • Drop-n-Stain™ EverBrite packaged in dropper bottles for easy dispensing • Available with or without DAPI • EverBrite™ Hardset available with far-red NucSpot® 640 nuclear counterstain, avoids cross-talk and UV photoconversion problems with DAPI
EverBrite™ Hardset Mounting Medium	23003-23004, 23016	
Drop-n-Stain EverBrite™ Mounting Medium	23008-23009	

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Products for Immunofluorescence and Western Blotting (continued)

Product	Cat. No.	Features
EverBrite TrueBlack® Hardset Mounting Medium	23017-23019	• The only mounting medium with autofluorescence quenching • Quenches as it hardens, with low background
CoverGrip™ Coverslip Sealant	23005	• Superior alternative to nail polish for coverslip sealing • Won't mix with aqueous mounting media
NucSpot® Nuclear Stains	40083... 41038	• Green to near-IR fluorescent nuclear counterstains for fixed cells or tissue sections • Nuclear-specific, unlike TOTO®, TO-PRO®, or SYTOX® dyes
RedDot™2 Far Red Nuclear Counterstain	40061	• Far-red nuclear dye for the Cy®5 channel • Better nuclear specificity compared to Draq®7
Live-or-Dye™ Fixable Viability Stains	32002... 32018	• Fixable dead cell stains compatible with downstream immunofluorescence staining • Exclude dead cells from flow cytometry analysis • Suitable for microscopy
Live-or-Dye NucFix™ Red	32010	• Fixable nuclear dead cell stain • Exclude dead cells from flow cytometry analysis • Suitable for microscopy
VersaBlot™ Total Protein Normalization Kit	33025, 33026	• Superior linearity for western normalization compared to housekeeping proteins • Reversible prestain for downstream multi-color western blot analysis • Highly sensitive protein quantitation on PAGE gels (≤ 1 ng) or western membranes • Easily label purified proteins or cell lysates before SDS-PAGE • Near-infrared fluorescence for Typhoon® or Odyssey®
Peacock™ Prestained Protein Marker	21530	• Protein ladders for SDS-PAGE with blue and red visible bands ranging from 8 kDa to 245 kDa • Ladders also fluoresce in the far-red, convenient for fluorescent western blotting
Peacock™ Plus Prestained Protein Marker	21531	
Mini-Cell Scrapers	22003	• Collect cells from 96-well to 24-well plates • Polypropylene, sterile, disposable
4X Protein Loading Buffer with Orange Tracking Dye	40136	• Convenient buffers, blocking agents, and accessories for immunofluorescence or western blotting
1X PBS (2L) Buffer Powder Packets	22033	
Fixation Buffer	22015	
4% Paraformaldehyde in PBS, Ready-to-Use Fixative	22023	
Permeabilization Buffer	22016	
Permeabilization and Blocking Buffer	22017	
10X Fish Gelatin Blocking Agent	22010	
Fish Gelatin Powder	22011	
30% Bovine Serum Albumin Solution	22014	
Tween®-20	22002	
Ponceau S Solution	22001	
Super^{HT} Pap Pen 2.0	23023, 23024	

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Please visit www.biotium.com to view our full selection of products featuring bright and photostable fluorescent CF® Dyes, including primary antibody conjugates, streptavidin, phalloidin, and other bioconjugates, as well as conjugates of biotin, HRP, AP, R-PE, APC, and PerCP.

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