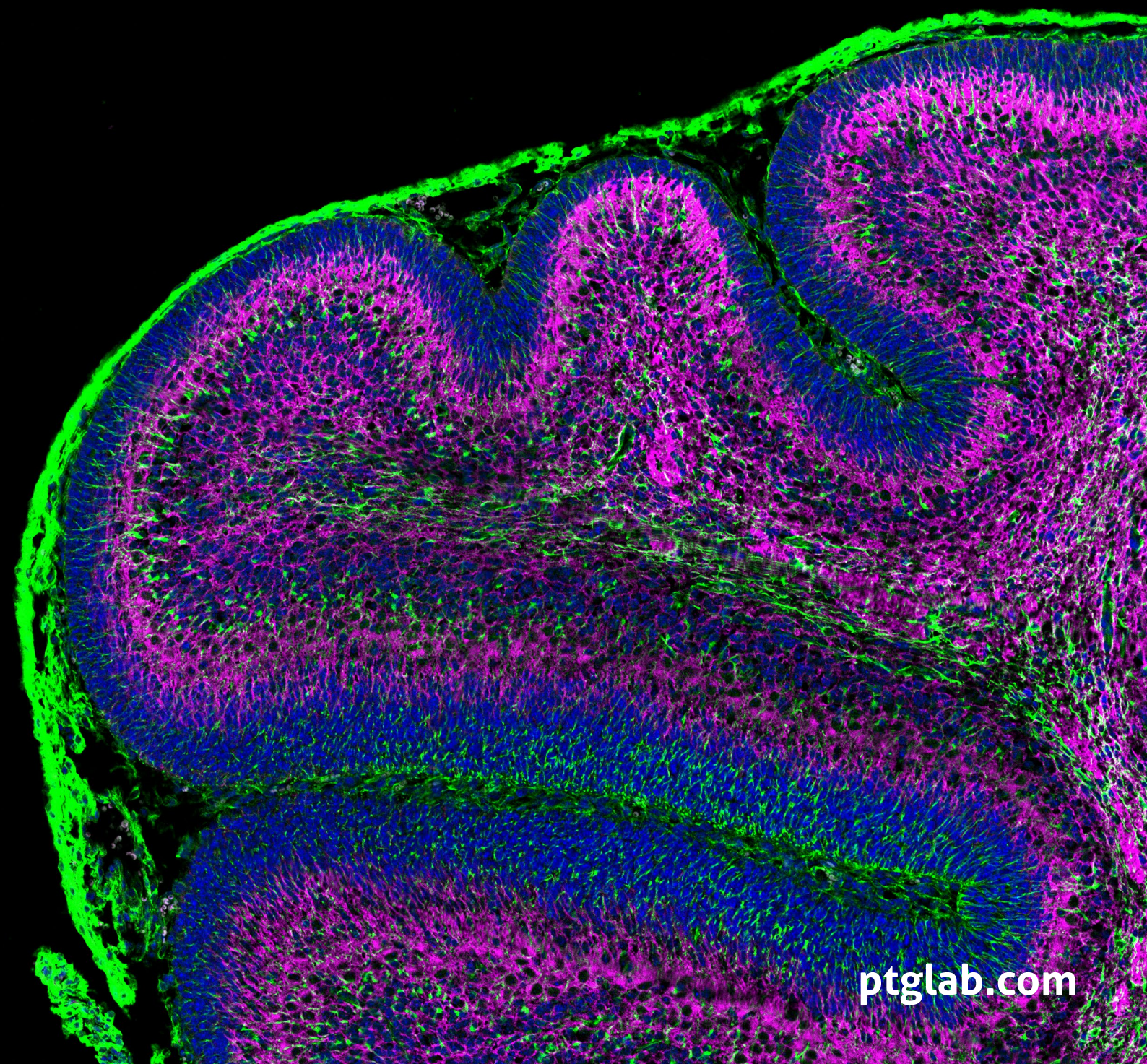
proteintech®

The Complete Guide To

Multiplex Immunofluorescence



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Inside This Guide

Your complete resource for multiplex immunofluorescence, from getting started and technology selection to supporting reagents, experimental workflows and troubleshooting.

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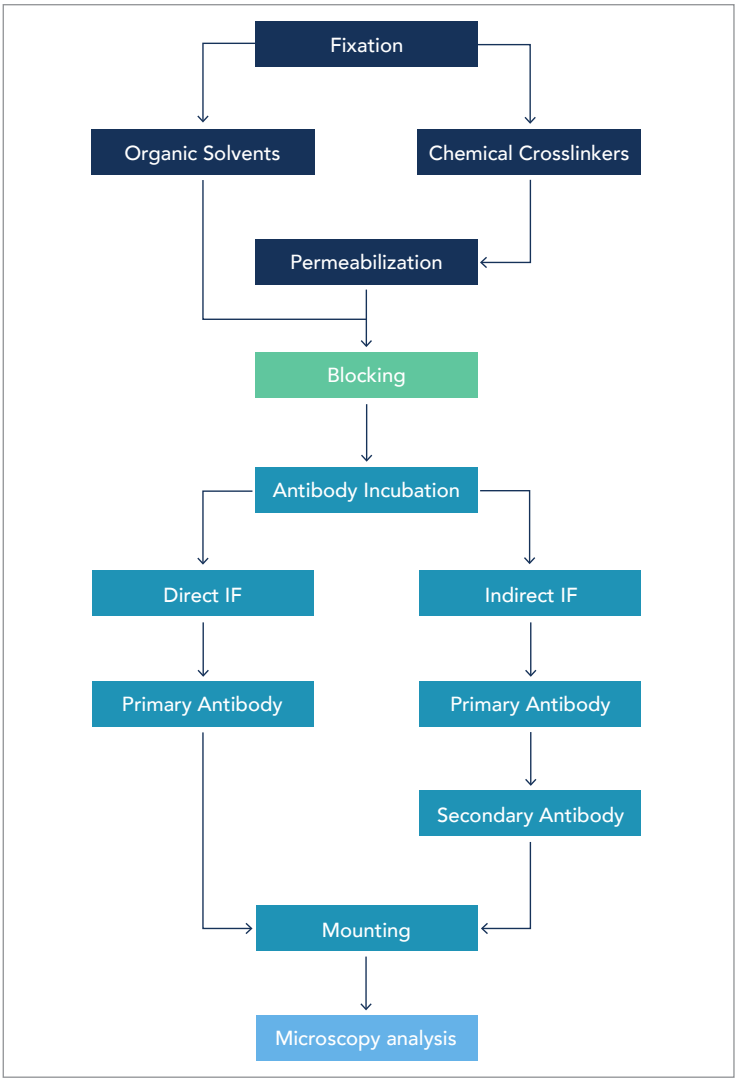
Conventional Immunofluorescence

In conventional immunofluorescence (IF), all markers are stained in a single round and imaged simultaneously, either by direct or indirect staining.

Conventional IF is typically limited to the visualization of 4–5 proteins per sample due to technical constraints, including:

- spectral overlap between fluorophores
- antibody cross-reactivity
- photobleaching
- tissue autofluorescence

As a result, conventional IF provides only a narrow snapshot of cellular composition, cell-cell interactions, activation states, etc., which are crucial to study tissue function.



What Is Multiplex Immunofluorescence?

The term "multiplexing" refers to techniques that use dyes or labeled antibodies for the visualization and analysis of several molecular targets within a single sample, typically while preserving its spatial context.

Start your multiplex workflow with Able AI.

Find compatible antibodies, fluorophores, and labeling kits in seconds.

Try asking Able AI...

"Build a 5-color multiplex IF panel for human liver tissues."

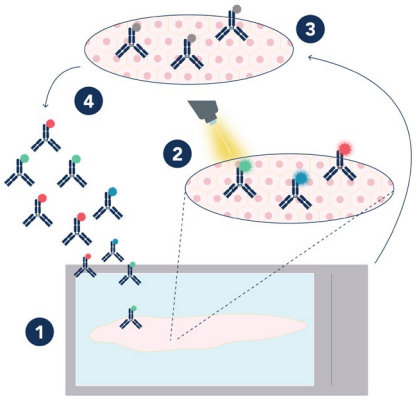
Advanced Multiplexing Technologies

To overcome the analytical limitations of conventional immunofluorescence (see p. 3) and better understand complex tissues, several advanced multiplex technologies have emerged (see p. 5). These methods greatly expand the number of detectable markers in a single sample – typically 10 to 60+, depending on the platform. This enables deeper insights into tissue organization, including the identification of diverse cell types and functional states.

Such technologies not only enhance our understanding of healthy tissues but also reveal critical features of diseases (e.g., tumors and their microenvironment), ultimately helping to better predict patient prognosis and support biomarker discovery.

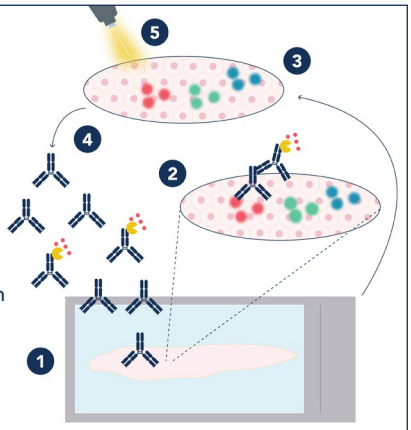
A. Fluorophore inactivation technologies (e.g., cycIF)

1. Fluorophore-labeled antibody staining
2. Imaging
3. Fluorophore bleaching or antibody removal
4. Repeat steps 1-3 with different fluorophore-labeled antibodies



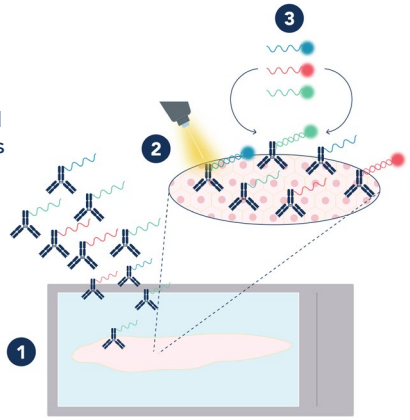
B. Enzyme-based technologies (e.g., TSA)

1. Primary antibody, horseradish peroxidase-coupled secondary antibody staining, and substrate addition
2. Enzymatic reaction
3. Antibody removal
4. Repeat steps 1-3 with different antibodies
5. Final all-in-one imaging



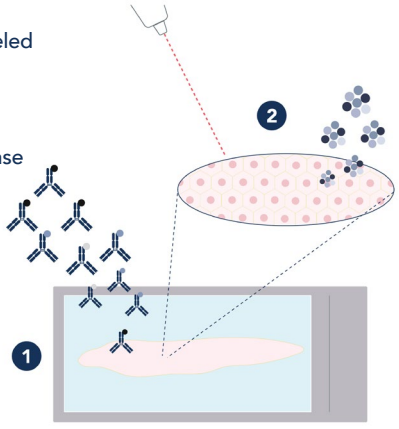
C. DNA-barcoding technologies (e.g., CODEX)

1. DNA-barcoded antibody staining
2. Hybridization of fluorophore-labeled DNA imager probes and subsequent imaging
3. Probe dehybridization
4. Repeat steps 2-3 with different imager probes



D. Metal-based technologies (e.g., IMC)

1. Metal isotope-labeled antibody staining
2. Laser ablation of tissue and antibodies to release isotopes and mass spectrometry



Choosing the Right Multiplex Technology

Fluorophore inactivation technologies

Method	Antibody Conjugation	Signal Removal	Maximum Marker Capacity
Standard single-round IF	Fluorophore/Enzyme	-	4-5 (≥15 with spectral unmixing methods)
CycIF/t-CycIF/CellDive	Fluorophore	H_2O_2 and light inactivation	~60
IBEX	Fluorophore	$LiBH_4$-based bleaching	~65
MxIF	Fluorophore (cyanine dyes)	Alkaline oxidation with a H_2O_2/NaOH solution	~60

Enzyme-based technologies

Method	Antibody Conjugation	Signal Removal	Maximum Marker Capacity
TSA	HRP (+ fluorescent tyramide dyes)	Antibody stripping	~8-16 (can be combined with t-CycIF)
SIMPLE	HRP (+ alcohol-soluble red peroxidase substrate AEC)	Removal of AEC with ethanol + Antibody stripping (Acidified $KMnO_4$ or citrate buffer)	~12

DNA-barcoding technologies

Method	Antibody Conjugation	Signal Removal	Maximum Marker Capacity
CODEX	DNA barcode	Dehybridization of imaging strand	~60
DEI	DNA barcode	Dehybridization of imaging strand	8
DSP	DNA barcode with UV-photocleavable linker	Focused UV light releases DNA barcodes from the region of interest; they are collected and quantified by NGS	96
Immuno-SABER	DNA barcode	Dehybridization of imaging strand	~10

Metal isotope-based technologies

Method	Antibody Conjugation	Signal Removal	Maximum Marker Capacity
IMC	Metal isotope	Only one imaging cycle – laser ablation destroys the tissue	>40
MIBI	Metal isotope	Only one imaging cycle – Ion beam sputtering destroys the tissue	40-100

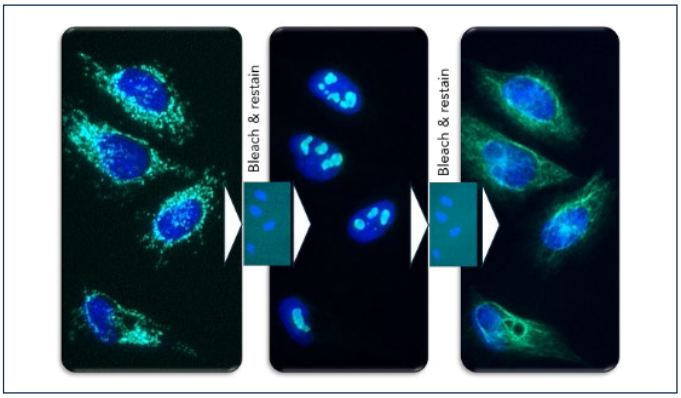
Fluorophore Inactivation Technologies

For multiplex IF methods that are based on fluorophore-conjugated antibodies, signal removal occurs mostly via fluorophore inactivation.

Fluorophore inactivation is achieved by fluorophore quenching/bleaching or fluorophore oxidation.

Multiplex IF methods that use fluorophore-labeled antibodies include:

- CycIF/t-CycIF using H_2O_2 and light-induced photobleaching
- MxIF using alkaline oxidation of cyanine dyes
- IBEX using $LiBH_4$-based bleaching



(Above) Cyclic IF: PFA-fixed HeLa cells were stained with mouse IgG1 anti-HSP60 antibody (66041-1-Ig, Cycle 1) labeled with FlexAble 2.0 CoraLite® Plus 750 Kit (KFA504), imaged and bleached with 4.5% H_2O_2 + 24 mM NaOH in PBS (60 min at RT, light). The next two cycles were performed accordingly using mouse IgG1 anti-B23 antibody (60096-1-Ig, Cycle 2) and mouse IgG1 anti-vimentin antibody (Sigma V6630, Cycle 3) labeled with FlexAble 2.0 CoraLite® Plus 750 (KFA504), respectively. Cell nuclei were stained with DAPI (blue).

Advantages	Considerations
• Cost-effective and accessible • Gentler signal removal than with antibody stripping methods • High multiplexing capacity	• Antibodies remain bound after one cycle and can sterically interfere with antibody binding in the next cycle**, lowering signal quality** • Many cycles can damage the tissue or epitopes • Technical complexity: incomplete bleaching and signal carryover, tissue autofluorescence, precise image alignment required to merge data, requires optimization

An alternative way to remove the signal is antibody stripping, which removes the whole antibody – not only the fluorophore. This solves the problem of steric hindrance by residual bound antibodies seen with fluorophore inactivation methods. However, harsh stripping procedures can damage binding epitopes on the tissue. An example of this is the enzyme-based method TSA (see p. 11).

Need help building your multiplex panel?

Ask Able AI for validated reagent recommendations and workflow guidance.

Supporting Reagents for Fluorophore Inactivation Technologies

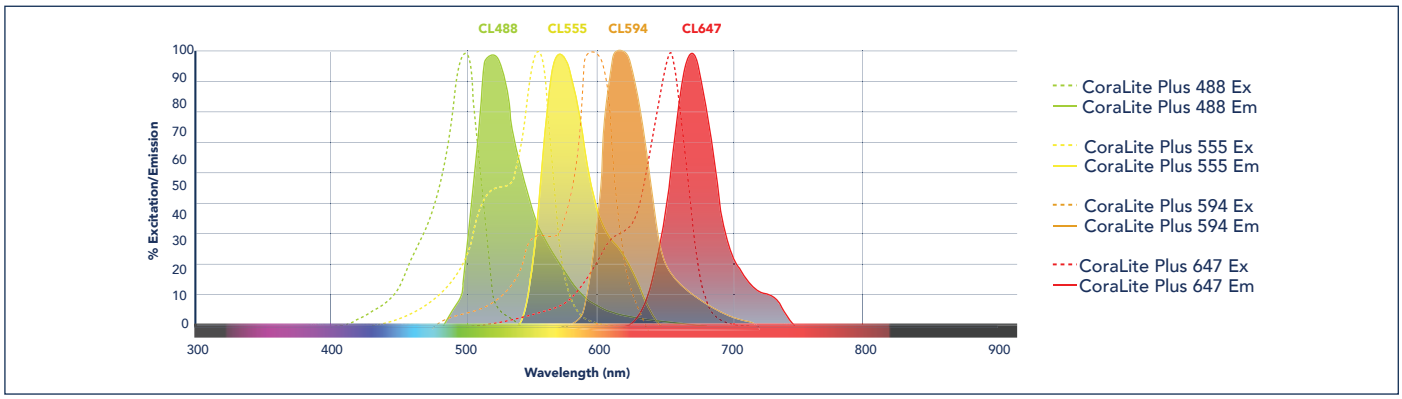
Directly Conjugated Antibodies

Proteintech's popular monoclonal, polyclonal, and recombinant antibodies are available directly conjugated to multiple fluorescent dyes: **CoraLite® Plus 488, CoraLite® Plus 555, CoraLite® Plus 594,** and **CoraLite® Plus 647.**

Proteintech has the largest in-house conjugated portfolio on the market. Each fluorophore can be used simultaneously in co-localization studies due to minimal between their fluorescence spectra.

Discover More

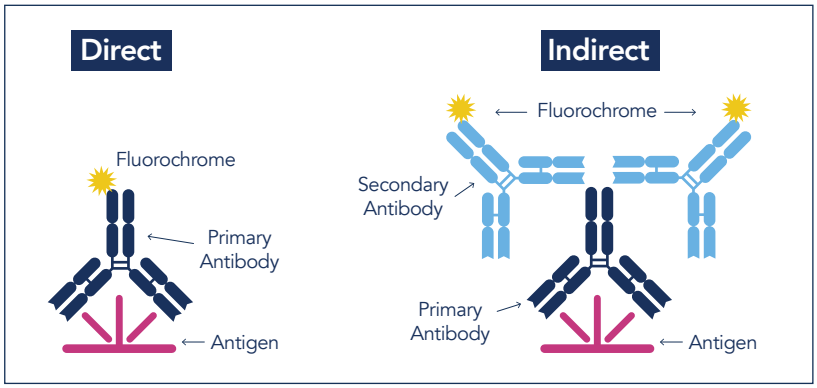


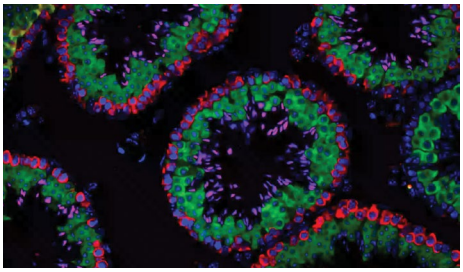


(Above) Fluorescence spectra of Proteintech CoraLite® Plus Fluorescent Dyes

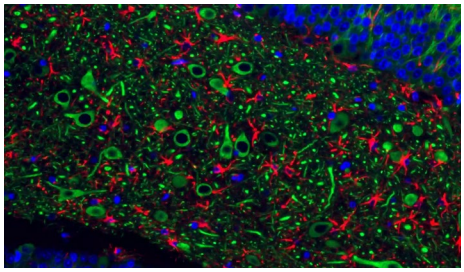
Benefits:

- Faster protocol
- High-resolution validation data
- Excellent brightness
- High photostability
- Easy to multiplex
- High degree of labeling

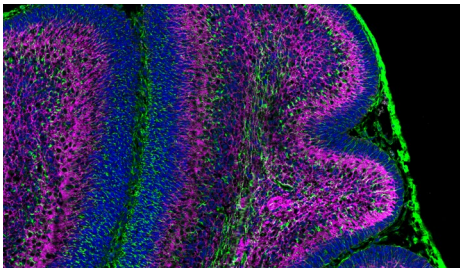




(Above) Mouse testis tissue stained using CoraLite® Plus 488 BOULE antibody (green), CoraLite 594 DAZL antibody (red) and CoraLite® Plus 647 TNP1 antibody (magenta).



(Above) Mouse brain stained with CoraLite® Plus 488 MAP2 antibody (green), CoraLite® 594 GFAP antibody (red) and CoraLite® Plus 647 AQP4 antibody (magenta).



(Above) Neonatal mouse cerebellum stained with CoraLite® 488 Beta III tubulin antibody (magenta, pseudocolor) and GFAP antibody (green).

Primary and Secondary VHHs (Nanobodies)

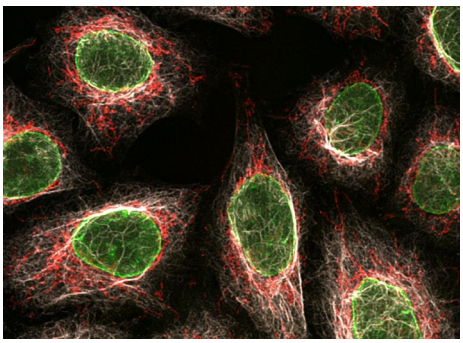
VHHs (Nanobodies) are recombinant alpaca-derived antigen-binding fragments with a size of just ~10% of conventional IgGs**, and are** available pre-conjugated with CoraLite® Plus dyes (e.g., 488, 555, 647, or 750), ATTO dyes, biotin, or as unconjugated primary or secondary reagents.

Primary VHH Targets	Secondary VHH Targets
Anti-tag VHHs: • GFP (GFP-Booster) • RFP (RFP-Booster) • HA tag • And more **Immuno-oncology, cytokine, and growth factor VHHs:** • FLT3 • PD-1/PD-L1 • TIM-3 • IL-6 • And more	• Human IgG • Mouse IgG1, IgG2a, IgG2b, IgG3 • Rabbit IgG

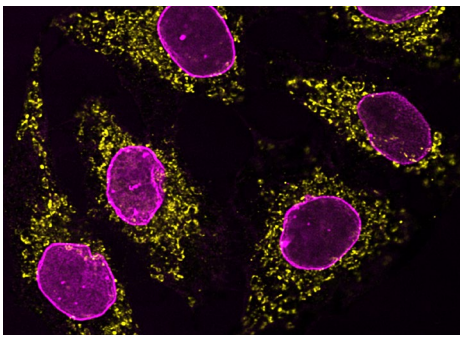
Discover More

Benefits:

- Better tissue penetration
- No cross-reactivity and high subclass specificity
- Higher resolution in imaging due to minimal epitope-label displacement
- Monovalency: no epitope cross-linking or artificial receptor clustering in live-cell applications
- High lot-to-lot consistency

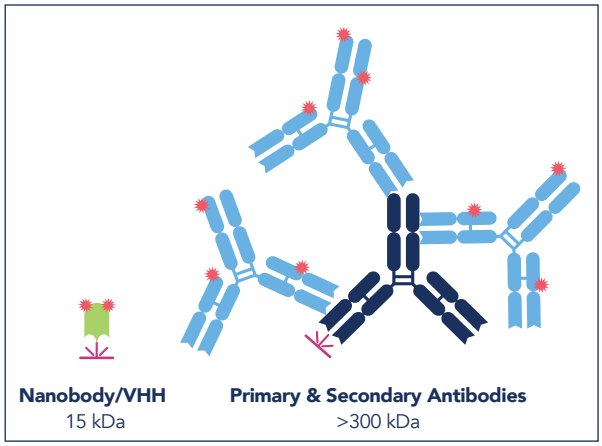


(Above) Multiplexed immunostaining of HeLa cells with three subclass-specific alpaca anti-mouse Nano-secondaries. Gray: Mouse IgG1 anti-vimentin + alpaca anti-mouse IgG1 VHH Alexa Fluor® 647. Green: Mouse IgG2b anti-lamin + alpaca anti-mouse IgG2b VHH Alexa Fluor® 488. Red: Mouse IgG3 anti-MOT + alpaca anti-mouse IgG3 VHH Alexa Fluor® 568. Scale bar, 10 µm. Images were recorded at the Core Facility Bioimaging, LMU Munich.



(Above) Immunofluorescence analysis of HeLa cells co-stained with mouse IgG1 anti-HSP60 antibody (66041-1-Ig) and mouse IgG2b anti-lamin A/C antibody followed by Nano-secondary® alpaca anti-mouse IgG1, recombinant VHH, CoraLite® Plus 555 (smsG1CL555-1, yellow) and Nano-secondary® alpaca anti-mouse IgG2b, recombinant VHH, CoraLite® Plus 647 (smsG1CL647-1, magenta). Images were recorded at the Core Facility Bioimaging, LMU Munich.

(Right) Epitope-to-label displacement: Schematic comparison of a nanobody (green) conjugated to fluorescent dyes (red) vs. a conventional primary antibody (dark blue) and secondary antibodies (light blue) conjugated to fluorescent dyes. Epitope is shown in pink.



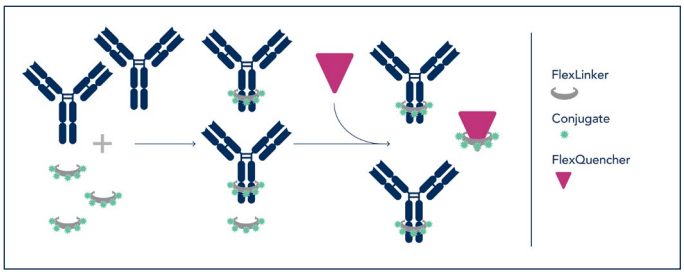
Antibody Labeling and Conjugation Kits

Direct staining enables same-species multiplexing in IF and makes workflows simpler and faster. If a pre-conjugated antibody is not available or not in the right color, a convenient solution is to label the antibody with Proteintech's simple Antibody Labeling Solutions.

FlexAble 2.0 Kits use a high-affinity site- and species-specific peptide linker to label IgGs with fluorophores, enzymes, biotin, or oligos in a simple 15-min protocol that does not require chemical expertise.

Benefits:

- Works with any antibody buffer and concentration
- Site-specific labeling
- Works with antibody input from as little as 0.5 μg IgG

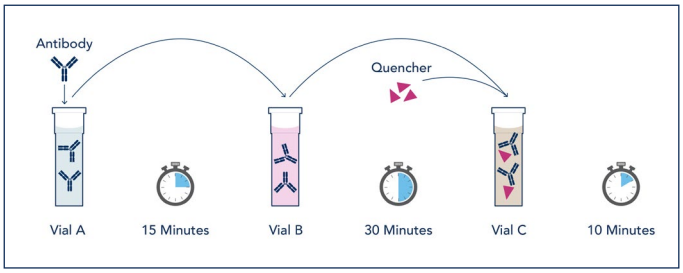


(Above) FlexAble workflow. FlexAble uses a high-affinity, non-covalent FlexLinker, which binds tightly to the IgG within minutes. Unbound FlexLinker is neutralized by adding FlexQuencher. The reaction yields a well-defined and fully functional IgG.

ChemBright Kits use maleimide conjugation to covalently conjugate an antibody or other protein, in PBS-only buffer, to a fluorophore or biotin. It is a simple 6-step, 55-minute protocol that does not require column purification, so there is no antibody loss during the conjugation process.

Benefits:

- Reduction-free maleimide conjugation
- No purification needed
- Compatible with any lysine-containing antibody, protein, or peptide

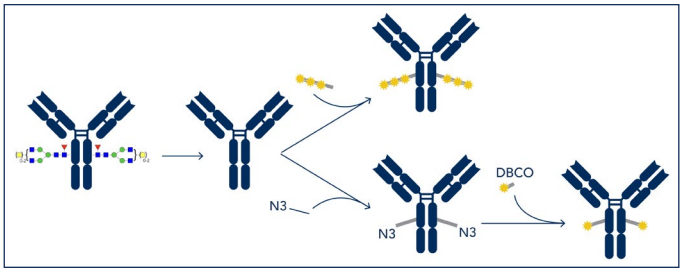


(Above) ChemBright workflow. An antibody modifier adds sulfhydryl groups at the lysines on the antibody or protein, which react with the maleimide dye to form covalent thioether bonds. The reaction is quenched to prevent further modification and conjugation.

ZymeBright Kits use an enzymatic method to conjugate fluorescent dyes or DBCO-modified conjugates to the Fc fragment of antibodies site-specifically. It results in a stable, covalently conjugated antibody. Proteintech's fluorescent ZymeBright Kits do not require purification.

Benefits:

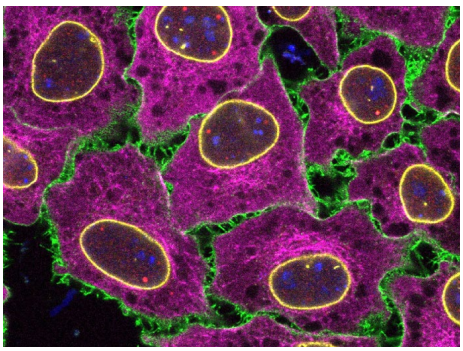
- Covalent, site-specific conjugation
- Conjugate fluorophores or any DBCO-modified molecule
- Available for bulk conjugations



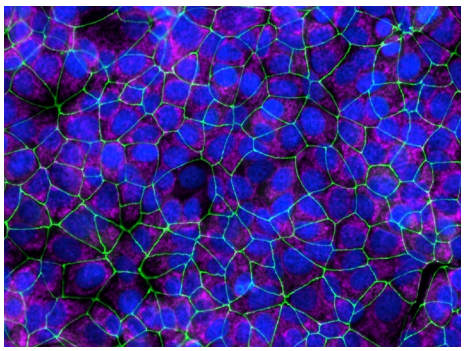
(Above) The ZymeBright workflow. 1. Modify Fc fragment to add two conjugation sites. 2. Enzymatically conjugate one pre-conjugated linker per conjugation site. 3. Or enzymatically conjugate one azide-modified linker per conjugation site. 4. Conjugate DBCO-modified conjugates to linker via click chemistry.

Comparison of Proteintech's Antibody Labeling Solutions

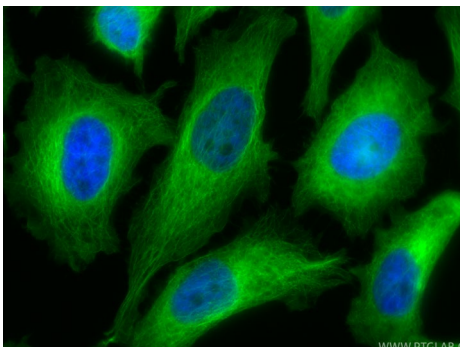
	FlexAble 2.0	ChemBright	ZymeBright
Colors and Labels	CoraLite® Plus 405, 488, 555, 647, 750, FITC, HRP, Biotin, Oligos	CoraLite® Plus 405, 488, 594, 647, 700, 750, FITC, Biotin	PE, APC, PE-Cyanine7, APC-Cyanine7, Biotin, DBCO-Ready
Chemistry	Affinity Linker	Covalent (Maleimide)	Covalent (Enzymatic)
Buffer	Any buffer	PBS-only, < 0.1% BSA	See website for buffer compatibility
Min. Amount	0.5 μg/reaction	100 μg/reaction	100 μg/reaction
IgG type	Isotype-specific kits	Any IgG	Rabbit IgG, Rat IgG1 and IgG2a, Human IgG1-4, or Mouse IgG1 & IgG3
Duration	15 min	60 min	Overnight
Labeled Ab Storage	Not recommended (use right away)	1 year at 4°C	1 year at 4°C
Site-specificity	Yes	No	Yes
Degree of Labeling	Defined	Variable	Defined



(Above) Immunofluorescence of HeLa cells using five FlexAble-labeled rabbit antibodies: PFA-fixed cells were stained with anti-Ki-67 Ab (MA5-14520, blue) + FlexAble KFA506, anti-CD147 Ab (11989-1-AP, green) + FlexAble KFA501, anti-Lamin B1 Ab (12987-1-AP, yellow) + FlexAble KFA502, anti-Coilin Ab (10967-1-AP, red) + FlexAble KFA503, and anti-Tubulin Ab (80713-1-RR, magenta) + FlexAble 2.0 CoraLite® Plus 800 (coming soon). Confocal images were acquired with a 60x oil objective.



(Above) Immunofluorescence of MCF-7 cells using ChemBright-conjugated antibodies: PFA-fixed cells were stained with anti-ZO-1 (21773-1-AP) labeled with ChemBright Kit (PK30030, green), and anti-TOM20 (80501-1-RR) labeled with ChemBright Kit (PK30031 magenta). Cell nuclei were stained with DAPI (blue).

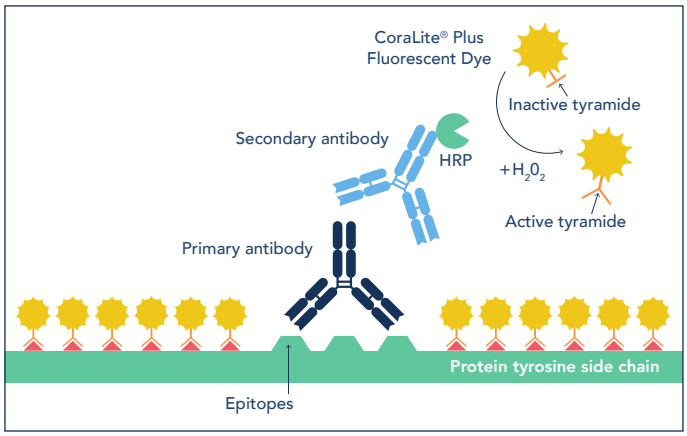


(Above) Immunofluorescence of HeLa cells using ZymeBright-conjugated antibodies: Beta Tubulin Recombinant Monoclonal Antibody (CL488-80713) was conjugated to CoraLite Plus 488 using ZymeBright Site-specific Antibody Conjugation Kit (DOL = 6) and used for immunofluorescent analysis of (4% PFA) fixed HeLa cells at dilution of 1:100.

Enzyme-Based Technologies

The most widely used multiplexed enzyme-based technology is Tyramide Signal Amplification (TSA). TSA uses HRP-conjugated antibodies and tyramide fluorophores. Upon HRP-catalyzed activation, tyramides are converted into highly reactive radicals that covalently bind to nearby tyrosine residues at the target site.

TSA workflows also include antibody stripping between staining cycles, while the fluorescent signal remains permanently anchored in the tissue due to covalent deposition. After the final cycle, all markers are imaged in a single acquisition step.



Advantages	Considerations
• Provides several 100-fold signal amplification compared to conventional IF → Great to detect low-abundance targets • Highly suitable for tissue multiplexing: TSA dyes can be sequentially added through cyclical antibody stripping and re-probing • Versatile multiplex options: Can be combined with several other technologies, e.g., traditional primary and secondary staining, FlexAble 2.0.	• Accurate measurement of co-localized targets may be challenging and require optimization (strong signal amplification can complicate interpretation) • Marker capacity for multiplexing is limited • Challenging to measure thicker samples

Achieve up to 5-plex imaging with

CoraLite® Plus-Tyramide Reagents and Detection Kits

- Up to 100x more sensitive than indirect staining methods
- Achieve publication quality data at the first attempt
- Wide dynamic range enabling the detection of low-abundance targets
- Easy multiplexing with no limitations on primary antibody host

View all TSA Reagents



Supporting Reagents for Enzyme-Based Technologies

CoraLite® Plus-Tyramide Reagents

CoraLite® Plus Reagents can be used with any HRP-conjugated secondary antibody for multiplexed IHC, ICC, or ISH experiments.

TSA Detect Kits

TSA Detect Kits are complete fluorescent IHC workflow solutions for tissue multiplexing. They include 3 or 4 tyramide reagents, DAPI nuclear stain, Multi-rAb® Polymer-HRP Recombinant Secondary Antibody, quenching buffer, blocking buffer, and amplification buffer.

CoraLite® Plus-Tyramide Reagents

Kit	Catalog No.
CoraLite® Plus 488-Tyramide Reagent	PR30022
CoraLite® Plus 555-Tyramide Reagent	PR30023
CoraLite® Plus 594-Tyramide Reagent	PR30024
CoraLite® Plus 647-Tyramide Reagent	PR30025

TSA Detect Kits

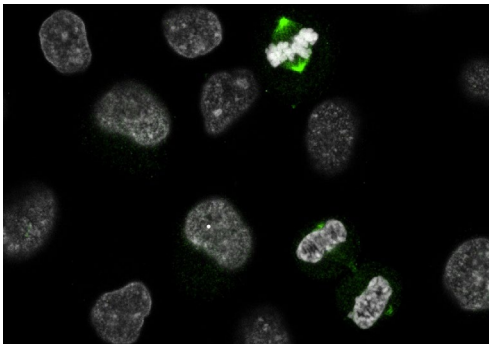
Kit	Catalog No.
4-Color TSA Detect Kit for Rabbit/ Mouse Primary Antibodies	PK10032
4-Color TSA Detect Kit for Rabbit Primary Antibody	PK10033
5-Color TSA Detect Kit for Rabbit/ Mouse Primary Antibodies	PK10034
5-Color TSA Detect for Rabbit Primary Antibody	PK10035

Benefits:

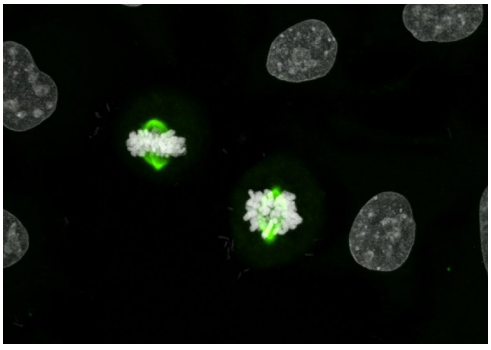
- Up to 100x more sensitive than indirect staining methods
- Ready-to-use, no pre-optimization needed
- Wide dynamic range enables detection of low-abundance targets
- Easy multiplexing with no limitations on primary antibody host

Discover More

Increased Sensitivity Compared to Indirect Staining Methods



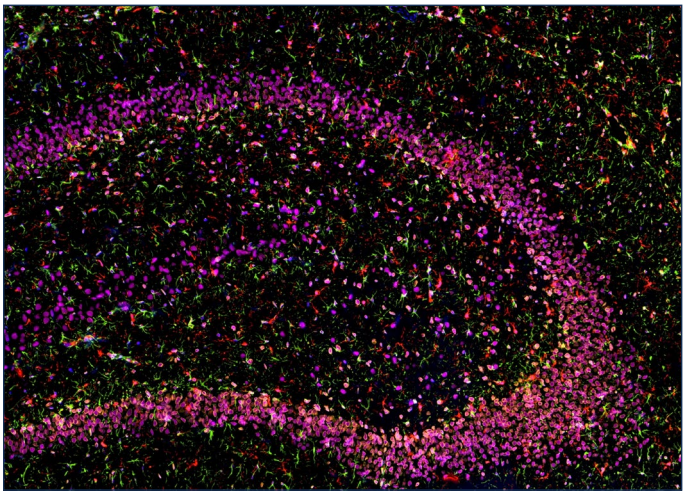
With Conventional Dye-Conjugated Secondary Antibody



With Multi-rAb Polymer HRP-Secondary and CoraLite® Plus 488-Tyramide Reagent

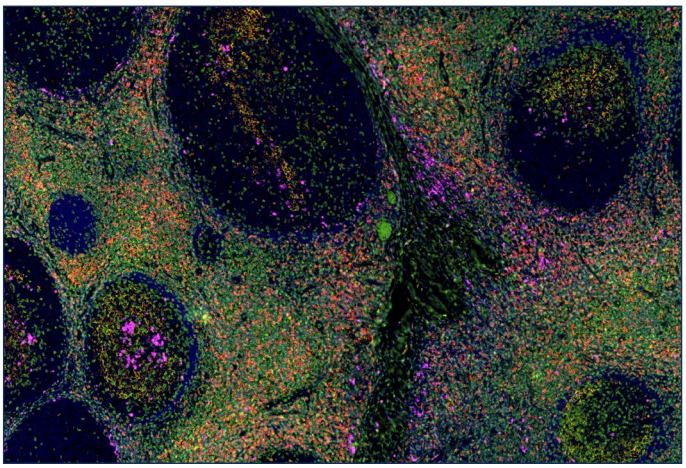
(Left) Confocal images of AURKA (green) in HeLa cells. DNA is stained with DAPI (gray). Detection with the tyramide reagent dramatically increases sensitivity and allows for better visualization of AURKA co-localization with intracellular DNA.

Achieve High-Quality Multiplexed Data

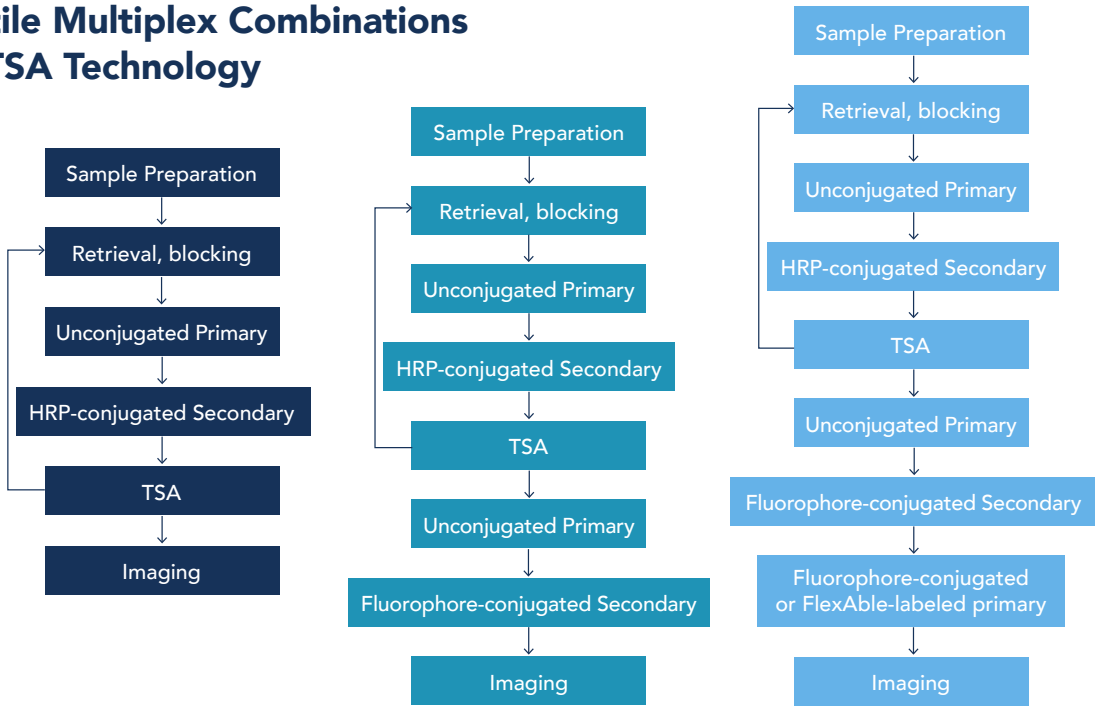


(Left) FFPE rat brain tissue tissue was stained with Lamin B1 monoclonal antibody (66095-1-Ig) at 1:2,000 followed by detection with Multi-rAb® Polymer HRP-Goat Anti-Rabbit/ Mouse Universal Recombinant Secondary Antibody (H+L). CoraLite® Plus 555-Tyramide Reagent was used for signal development (yellow). HIER was then performed in pH 9.0 Tris-EDTA buffer followed by staining with GFAP monoclonal antibody (60190-1-Ig, 1:2,0000). CoraLite® Plus 488-Tyramide Reagent was used for signal development (green). Similarly, IBA1 recombinant antibody (81728-1-RR, 1:2,000) and TDP-43 recombinant antibody (80002-1-RR, 1:3,200) were stained sequentially. CoraLite® Plus 594-Tyramide Reagent (red) and CoraLite® Plus 647-Tyramide Reagent (purple) were used for signal development, respectively. Cell nuclei (blue) were stained with DAPI.

(Right) FFPE human tonsil tissue was stained with CD23 monoclonal antibody (60208-2-Ig) at 1:8000 followed by detection with Multi-rAb® Polymer HRP-Goat Anti-Rabbit/ Mouse Universal Recombinant Secondary Antibody (H+L). CoraLite®Plus 555-Tyramide Reagent was used for signal development (yellow). HIER was then performed in pH 9.0 Tris-EDTA buffer followed by staining with CD3 monoclonal antibody (60181-1-Ig, 1:10000). CoraLite®Plus 488-Tyramide Reagent was used for signal development (green). Similarly, CD8a monoclonal antibody (66868-1-Ig, 1:20000) and CD138 monoclonal antibody (67155-1-Ig, 1:10000) were stained subsequently. CoraLite®Plus 594-Tyramide Reagent (red) and 647-Tyramide Reagent (purple) were used for signal development, respectively. Cell nuclei (blue) were stained with DAPI.



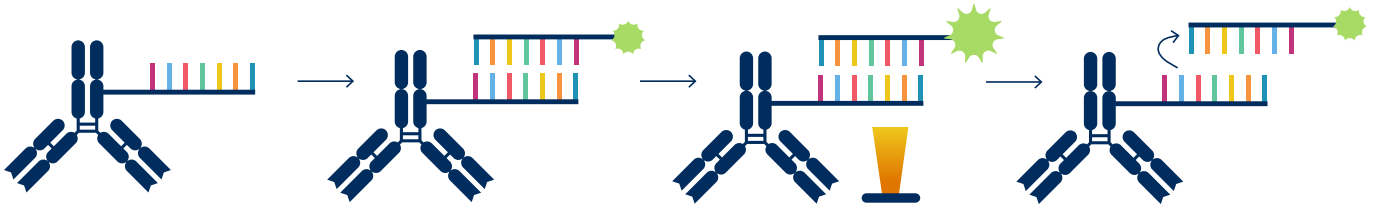
Versatile Multiplex Combinations With TSA Technology



DNA-Barcoding Technologies

DNA barcoding technologies such as CODEX, DEI, DSP, and Immuno-SABER use DNA-barcoded antibodies to enable highly multiplexed protein detection.

Co-detection by indexing (CODEX) and DNA Exchange Imaging (DEI) rely on cyclic hybridization of fluorescent imager strands to antibody-bound DNA barcodes, followed by imaging and barcode removal. DEI is a flexible strategy that can be implemented across a variety of fluorescence microscopy platforms, whereas CODEX integrates the same underlying principle into an automated imaging platform.



Digital Spatial Profiling (DSP) combines fluorescence imaging for tissue visualization and region-of-interest selection with barcode-based molecular detection. Photocleavable DNA barcodes are released from selected tissue regions and quantified either by nCounter digital counting or next-generation sequencing (NGS), rather than by iterative imaging cycles.

Signal amplification by exchange reaction (Immuno-SABER) enhances detection sensitivity by hybridizing DNA concatemers to antibody-bound DNA barcodes, creating repeated binding sites for fluorescent DNA imagers, which generates an amplified signal. The related Exchange-SABER approach extends this concept to highly multiplexed imaging by enabling sequential exchange of fluorescent imager strands.

Advantages	Considerations
• Very high multiplexing capacity, enabling simultaneous detection of tens to hundreds of targets • Can enable multimodal workflows, combining imaging information with sequencing-based barcode quantification (e.g., DSP) • Greater flexibility in signal assignment, allowing targets to be distinguished through unique DNA barcodes rather than distinct fluorescent labels	• Higher experimental complexity and cost • Some techniques require proprietary reagents or instruments (e.g., CODEX) • Signal amplification is limited in many direct hybridization-based approaches • More demanding computational image processing and data analysis • Fluorescence-based detection remains susceptible to autofluorescence and, in cyclic workflows, tissue degradation and image registration errors

Supporting Reagents for DNA-Barcoding Technologies

FlexAble Oligo-Ready

Antibody Labeling Kits

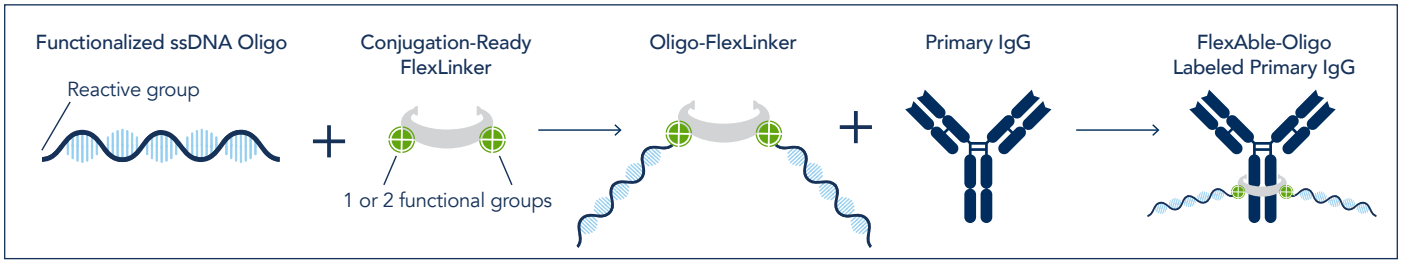
FlexAble Oligo-Ready Antibody Labeling Kits use our well-established FlexAble technology to label antibodies with oligonucleotides. High-affinity FlexLinkers (DBCO- or Azide-Ready) are conjugated with oligonucleotides via click chemistry. The resulting Oligo-FlexLinkers are then used to label antibodies in a non-covalent, site-specific manner (Fc-specific).

Benefits:

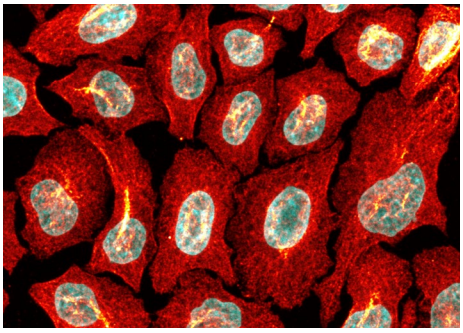
- High recovery rates of 60-80%
- Minimal buffer restrictions
- Site-directed with a defined degree of labeling (1 or 2)
- Small input requirements (from 0.5 µg IgG)
- Easy screening of various primary antibodies

Discover More

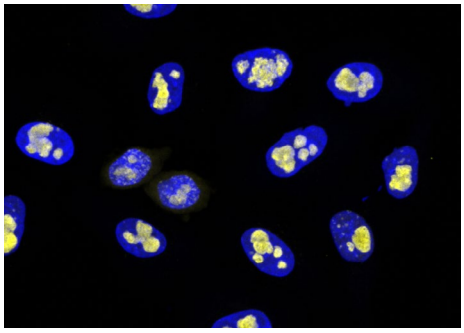
Overview of the Labeling Protocol



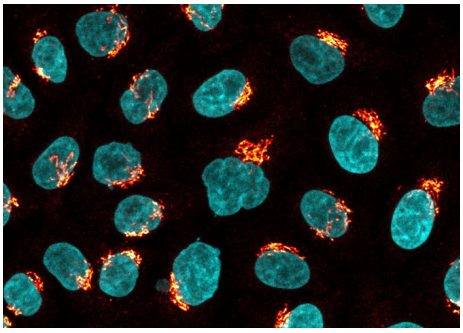
Hybridization Immunofluorescence Imaging



(Above) Immunostaining of HeLa cells with rabbit anti-Tubulin antibody (80713-1-RR, 1:500, hot orange), which was labeled with a 15-nt DNA oligonucleotide using the KFA151 Kit. Detection was performed by hybridization with an imaging DNA strand carrying ATTO643. Nuclei were stained with DAPI (cyan).



(Above) Immunostaining of HeLa cells with mouse IgG1 anti-B23 antibodies (60096-1-Ig, 1:500, yellow), which was labeled with a 15-nt DNA oligonucleotide using the KFA152 Kit. Detection was performed by hybridization with imaging DNA strands, carrying ATTO643. Nuclei are stained with DAPI (blue).



(Above) Immunostaining of HeLa cells with rabbit anti-GM130 antibody (11308-1-AP, 1:200, hot orange), which was labeled with a 15-nt DNA oligonucleotide using the KFA151 Kit. Detection was performed by hybridization with an imaging DNA strand carrying ATTO643. Nuclei were stained with DAPI (cyan).

CleanAb Antibody Conjugate Clean-Up

Conjugation CleanAb Kits use coated (magnetic) agarose beads designed for fast and easy removal of excess oligo or small molecules (e.g., dyes) from chemically conjugated antibodies.

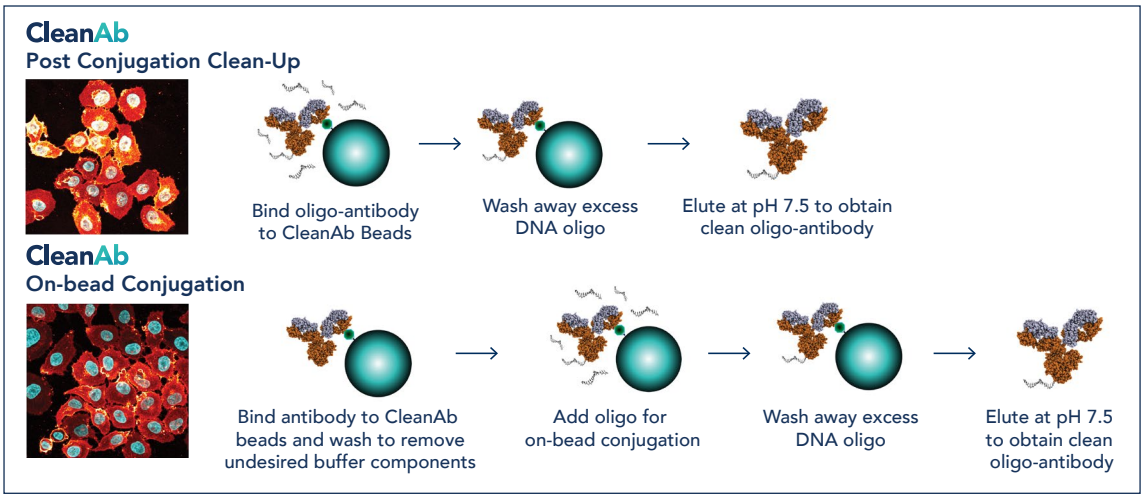
(Note: CleanAb is not required when using FlexAble 2.0, FlexAble Oligo-Ready, or ChemBright Kits.)

Benefits:

- Consistent, high input recovery (~80% on average)
- Gentle elution at pH 7.5 to preserve antibody integrity
- Reliable affinity and capacity across IgG species and subtypes
- Post-conjugation or on-bead conjugation workflow

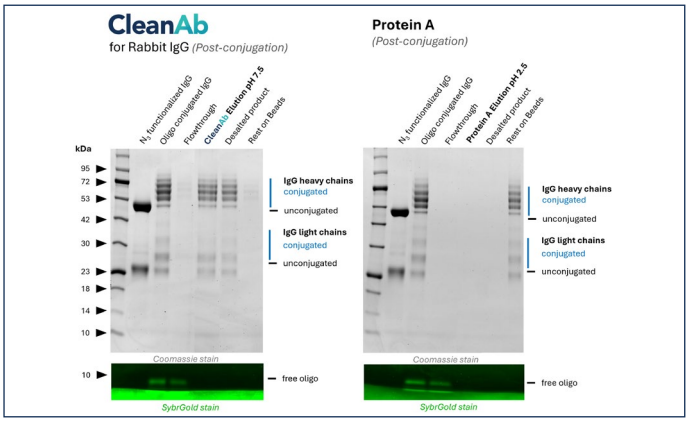
Discover More

Overview Of The CleanAb Workflows



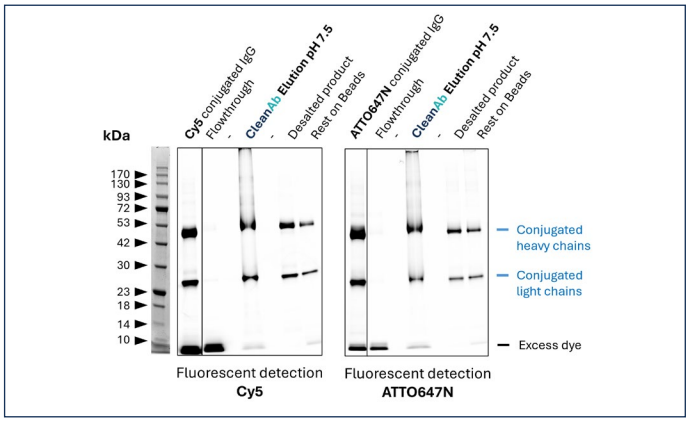
(Left) The scheme shows both post- and on-bead conjugation workflow with oligo conjugation as an example.

Oligonucleotide removal



(Above) Rabbit IgG (80713-1-RR) was conjugated with NHS-Azide and a DBCO-Oligo (lane 1+2) and then cleaned up with either CleanAb Kit (CK001) or Protein A (lanes 3-5, respectively). Both removed excess oligo as shown by SYBR Gold staining. But while IgG was eluted with ~70% recovery with CleanAb, Protein A IgG elution with low pH failed (lane 4). Only a little IgG remained on CleanAb beads, whereas most remained on Protein A beads (lane 6).

Fluorescent dye removal



(Above) Mouse IgG1 (66095-1-Ig) was conjugated with NHS-dyes (lane 1). Post-conjugation clean-up was done using CleanAb Kit (CK004, lanes 2-4). At least 95% of excess dye was successfully removed (lane 4). The conjugated IgG was eluted with high recovery (>70%) at neutral pH (lane 4). No IgG remained on beads (lane 7). Note: CleanAb Elution Buffer can interfere with SDS gels, which can make the elution appear smeared.

Basic Example Protocol for Cyclic IF

This basic protocol has been adapted from Lin et al., 2018; Kothurkar et al., 2024.

First Cycle:

1. Fix cells with 4% formaldehyde (use safety precautions) in PBS for 10 min at RT.
2. Wash cells three times with PBS.
3. Permeabilize cells with 0.5% Triton X-100 in PBS for 5 min at RT.
4. Wash cells two times with PBS.
5. Block cells with 4% BSA in PBS for 20 min at RT.
6. Incubate cells with fluorophore-labeled antibodies in 4% BSA in PBS for 60 min at RT.
7. Wash cells three times with PBS.
8. Stain cells with DAPI in PBS for 10 min at RT.
9. Image cells in PBS.

Next Cycles:

10. Wash cells three times with PBS.
11. Bleach cells using Option 1 or Option 2:

    Option 1 - Incubate stained cells in H_2O_2/NaOH: 4.5% H_2O_2 + 24 mM NaOH in PBS, prepare fresh and use safety precautions. Incubate for 60 min at RT in ambient light.

    Option 2 - Incubate stained cells in $LiBH_4$: Dissolve 10 mg $LiBH_4$ in 10 mL ddH_2O (= 1 mg/mL), incubate for 10 min at RT, filter through 0.22 µm filter and use within 4 h (Caution: $LiBH_4$ reacts vigorously with water). Incubate cells in $LiBH_4$ solution at RT in ambient light for a total of 180 min, replacing with fresh solution every 30 min.

12. Wash cells five times with PBS for 5 min each.
13. Image cells in PBS to confirm successful bleaching.
14. Incubate cells with fluorophore-labeled antibodies in 4% BSA in PBS for 60 min at RT.
15. Wash cells three times with PBS.
16. Image cells in PBS.

View Other Protocols and Application Notes

TSA protocol

IBEX protocol

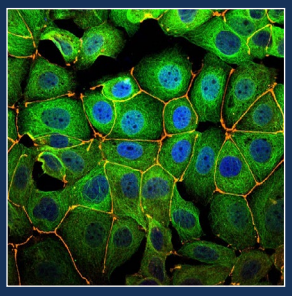
Multiplexing in expansion microscopy

Troubleshooting Tips

Problem	Troubleshooting Tips
Insufficient fluorophore inactivation (fluorophore-inactivation methods)	• H_2O_2 bleaching: 60 min should be sufficient for most fluorophores; for strong signals (e.g., from indirect staining) increase H_2O_2 concentration or incubation time. • $LiBH_4$ bleaching: Bleaching efficiency is fluorophore-dependent. For dyes that are relatively resistant to $LiBH_4$ alone, exposure to bright light during $LiBH_4$ treatment can improve signal removal. For persistent fluorescent signals, an additional heat-treatment step, such as heating in sodium citrate buffer, may also be effective and should be validated for the specific fluorophore and sample. • General tips: Use fresh solutions; use bright markers in later cycles.
Uneven staining	• Optimize sample preparation: avoid incomplete fix/perm. • Apply sufficient antibody solution to cover the sample and prevent drying.
Cell loss	• Optimize cell density for the experiment (not too high or too low). • Be gentle during washing. • Use an appropriate adhesion strategy for your tissue or adherent cells, e.g., charged slides or coating with collagen or poly-L-lysine. • Avoid storing samples for extended periods (post-permeabilization cells become more sensitive to washing).
Low signal	• Fluorophore-based methods: Try antibodies labeled with a brighter fluorophore or perform indirect IF (only one species/isotype of primary is possible per experiment). Alternatively, use TSA kits for very low-abundance targets to increase sensitivity. • Oligo-based methods: Specific methods have been developed in recent years to allow signal amplification, e.g., signal amplification by exchange reaction (Immuno-SABER). • General: Validate each antibody individually under the relevant fixation/retrieval condition. Optimize antibody concentration, incubation times, and imaging settings. Include a known positive control.
High background / nonspecific staining	• Ensure proper sample preparation (poor tissue morphology can also result in background). • Include biological positive and negative controls (e.g., overexpressing cells, knockout cells) and appropriate technical controls (e.g., singleplex, no primary, isotype controls). • Titrate every antibody to achieve an ideal signal-to-noise ratio. • Ensure proper stripping/bleaching to avoid signal carryover from cycle to cycle.
Poor reproducibility	• Standardize reagents, concentrations, incubation times, marker orders, and imaging settings. Document any deviations. • Use monoclonal or recombinant antibodies for higher lot-to-lot consistency. • Use automation where possible. • Use appropriate controls.

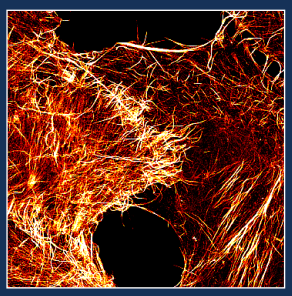
More information and tips can also be found in our general immunofluorescence imaging technical guide at **www.ptglab.com/support/immunofluorescence-protocol.**

Proteintech's Solutions for Multiplex Immunofluorescence



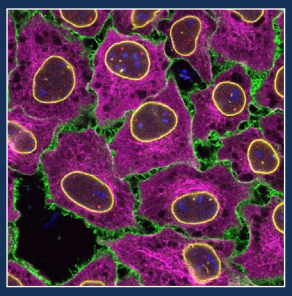
Directly Conjugated Primary and Secondary Antibodies

Choose from over 3,200 IF-validated directly labeled primary antibodies and secondary antibodies reactive to IgGs from 10 species, available in polyclonal, monoclonal, or recombinant formats and labeled with bright, photostable CoraLite® Plus dyes.



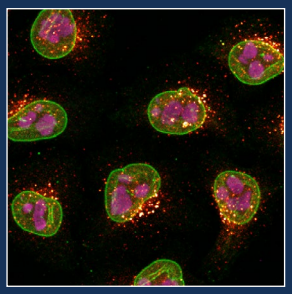
Primary and Secondary VHHs

VHHs (Nanobodies) are recombinant alpaca-derived antigen-binding fragments with a size of just ~10% of conventional IgGs available as primary and secondary reagents. Their small size, natural monovalency and high affinity make them excellent tools for super-resolution and live-cell imaging applications 'and for accessing difficult-to-reach targets.



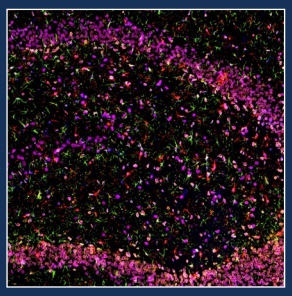
Labeling and Conjugation

From ultra-low input site-specific labeling with FlexAble 2.0 to high-input covalent conjugation with ChemBright, we offer flexible antibody labeling and conjugation solutions for every workflow.



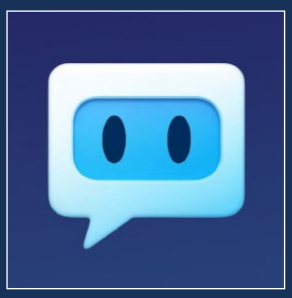
Oligo Conjugation Solutions

We support your entire oligo-conjugation workflow: FlexAble Oligo-Ready Kits let you generate up to three Oligo-FlexLinkers to produce ready-to-use antibody–oligo conjugates, with inputs from 0.5 µg per reaction up to 100 µg total. Conjugation CleanAb Kits provide fast and easy antibody clean-up after any oligo-conjugation protocol.



Tyramide Signal Amplification (TSA) Reagents and Kits

Choose from CoraLite® Plus-Tyramide Reagents in four colors for use with HRP-conjugated secondary antibodies or explore our complete 4- and 5-color TSA Detect Kits, which include 3-4 tyramide reagents, DAPI, and all other necessary components for a complete TSA assay workflow.



Able AI for Multiplex Imaging Assay Design

Able AI is a first-of-its-kind smart platform built to support scientists with reagent selection, literature search, and experimental workflow design.

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