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Imaging principles of life

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Sample preparation for light microscopy

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SCAN ME

Why Do We Need Labelling in Microscopy?

❗ Native biomolecules are invisible

- Proteins and structures are transparent and colorless under light microscopy.
- Labelling is necessary to detect and analyze them.

🔍 What Labelling Enables:

- ➢ Visualize the invisible: make specific proteins or structures detectable.
- ➢ Gain molecular specificity: distinguish between closely related components.
- ➢ Understand spatial organization: identify compartments and molecule distribution.
- ➢ Track dynamic processes: observe changes over time (e.g., mitosis, transport).
- ➢ Study molecular interactions: reveal colocalization and functional associations.

🧪 Common Labelling Techniques:

- ➢ Immunolabelling (antibody-based)
- ➢ Fluorescent protein tags (e.g., GFP, CFP…)
- ➢ Chemical dyes (e.g., DAPI, phalloidin)
- ➢ In situ hybridization (e.g., FISH)

Biological sample preparation for light microscopy

Biological samples are not stable. They are prone to degradation/contamination over the time. Necessary to fix the sample to preserve its structure and protect from contamination, before starting the visualization.

- Preserve the sample structure
  stabilize biological structures (proteins, membranes, organelles) from degradation - internal proteases, external factors.

- Enhances the contrast, visibility
  increase the binding sites for dyes and antibodies. Permeabilize the cell membrane for entering antibodies.

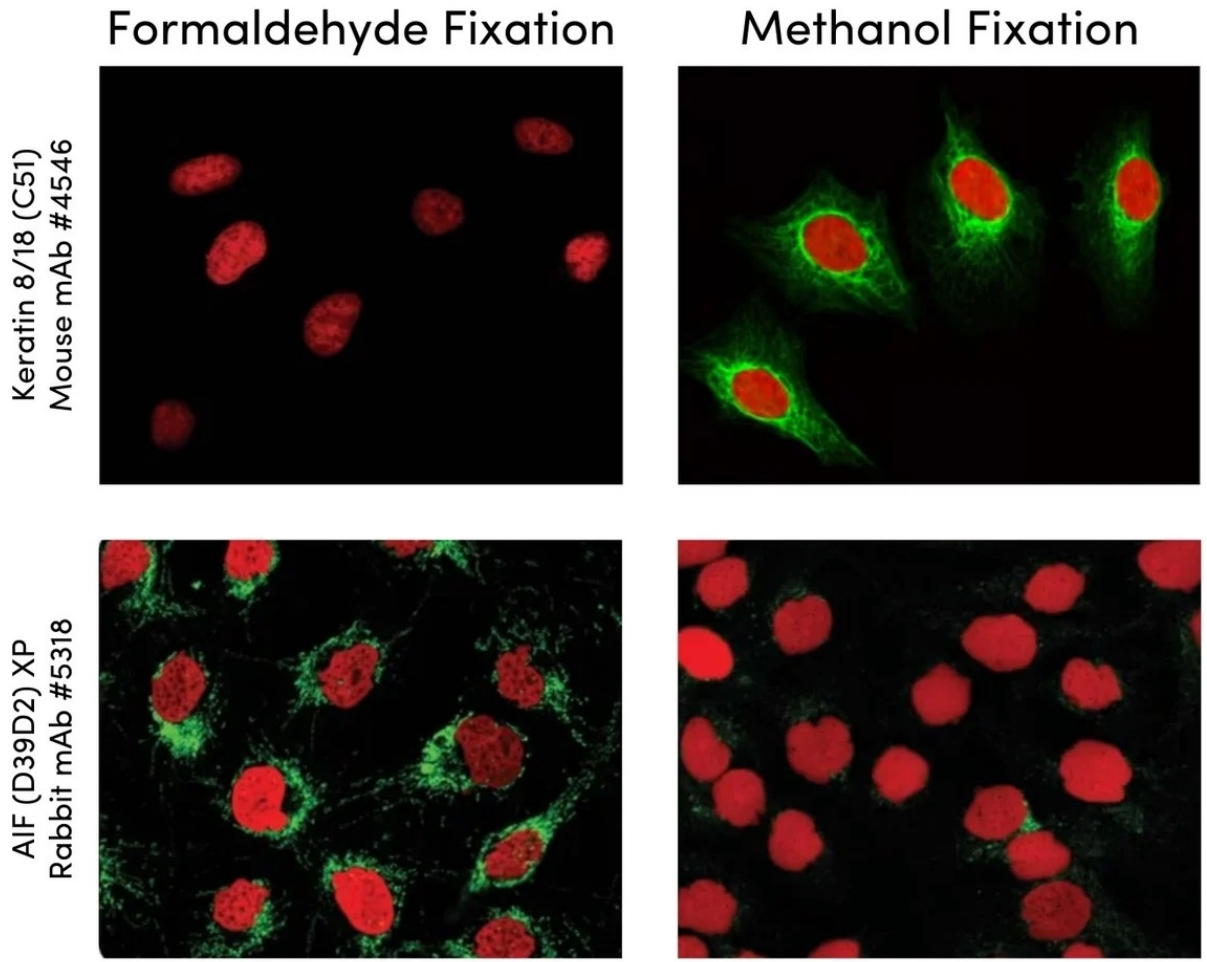
- Refraction index match - mounting media
  Mount to media for long term storage - prevent evaporation, contamination,
  Check refractive index of the media to match the objective requirements.

- Most common workflow
  Fixation, permeabilization, blocking, immunostaining with labelled antibodies, mounting

Common used Fixations

Method	Advantages	Disadvantages	Fluorescent proteins compatibility
Aldehyde based fixation (Formaldehyde, Glutaraldehyde)	Preserves structure well, compatible with most of immunostainings. Preserve fluorescence of fluorescent proteins.	Can cause crosslinking artifacts after prolonged period, may alter antigenicity	Yes, but prolonged fixation can reduce fluorescence
Alcohol-based Fixation (e.g., Methanol, Ethanol)	Rapid dehydration, good for cytoskeleton preservation.	Can cause cell shrinkage, may not preserve proteins well. Loose soluble proteins.	No, GFP fluorescence is reduced or lost
Acetone Fixation	Fast, preserves lipids, good for some immunostaining	Harsh, can extract proteins	No, GFP fluorescence is lost
Cryofixation (e.g., Liquid Nitrogen, High-pressure Freezing)	Best structural preservation, no chemical artifacts	Requires specialized equipment, costly	Yes, excellent preservation. Not for light microscopy.

Common used Fixations



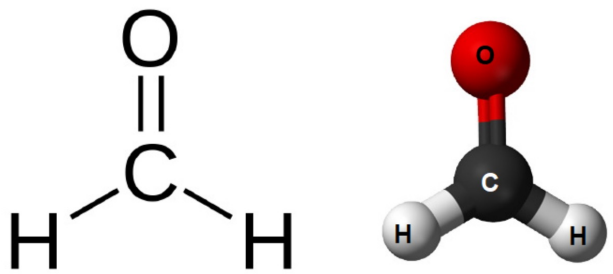
Confocal IF analysis of HeLa cells fixed with formaldehyde (left) or methanol (right) using Keratin 8/18 (C51) Mouse mAb #4546 (green, upper row) or AIF (D39D2) XP® Rabbit mAb #5318 (green, lower row). Red = Propidium Iodide (PI)/RNase Staining Solution #4087.

Formaldehyde CH_2O fixation

🔬 **Overview:**
Formaldehyde is a widely used fixative that crosslinks proteins by forming methylene bridges, preserving cellular structures while maintaining antigenicity. Most common 4% formaldehyde in PBS. Makes chemical crosslinks between lysine molecules of proteins.

✅ **Advantages:**
- Preserves cellular and tissue morphology effectively.
- Suitable for immunostaining and fluorescence microscopy.
- Maintains protein antigenicity for antibody labeling.
- Compatible with GFP and other fluorescent proteins (short fixation times recommended).

❌ **Disadvantages:**
- Can cause some crosslinking artifacts.
- Penetration into tissues is relatively slow.
- Can increase background fluorescence.
- Requires neutralization or washing to remove unreacted formaldehyde.
- Solution is unstable, often used with methanol as stabilizer.



Formaldehyde versus glutaraldehyde

	Formaldehyde	Glutaraldehyde
Chemical Type	Monoaldehyde (HCHO)	Dialdehyde (OHC–$(CH_2)_3$–CHO)
Cross-linking	Mild, reversible methylene bridges	Strong, stable cross-links
Penetration	Fast penetration, slow reaction	Slower penetration, fast reaction
Antigen Preservation	Good – suitable for immunostaining	May mask epitopes – limited use in immunostaining
Ultrastructural Detail	Limited preservation	Excellent preservation – ideal for EM
Fixation Reversibility	Partially reversible	Irreversible
Typical Use	Light microscopy, IHC, immunofluorescence	Electron microscopy, structural preservation

Formaldehyde CH_2O fixation

⚙️ **Protocol Recommendations:**
1. Use **4% formaldehyde** in **PBS or culture medium** (freshly prepared if possible).
2. Fix cells at **room temperature (10–15 min) or 4°C (20–30 min)**.
3. Wash with PBS after fixation to remove excess fixative.
4. Proceed with permeabilization (if required) before staining.

💡 **Best Practices:**
- Avoid over-fixation to preserve fluorescence.
- Store stock solution at **-20°C in aliquots** to prevent polymerization.

Permeabilization

Definition:
Permeabilization is the process of temporarily or permanently disrupting the cell membrane, allowing big molecules (e.g., non permeable dyes, drugs, proteins) to pass inside the cell. Together with fixation are crucial steps for successful staining.

Methods of Permeabilization:

1.Chemical Permeabilization 🧪

1. Use of detergents (e.g., Triton X-100, saponins, Tweens, NP-40 or other detergents)
2. Use organic solvents (e.g. methanol, acetone)

2.Mechanical Permeabilization ⚙️

1. Sonication (ultrasound waves)
2. Electroporation (short electrical pulses)
3. Microinjection (direct insertion of substances)

Permeabilization with detergents

What is detergent-based permeabilization?

Detergents are amphiphilic molecules (having both hydrophilic and hydrophobic parts) that **disrupt the lipid bilayer of cell membranes**, making them permeable to small molecules, antibodies, or stains.

How Detergents Permeabilize Cells?

1. Detergents **solubilize membrane lipids**, disrupting membrane integrity and creating pores.
2. This makes the membrane leaky and **allows molecules (e.g., dyes, antibodies, drugs) to enter the cell**.

Types of detergent

🧪 1. Ionic Detergents

•**Head group:** Carries a **net electric charge** (positive or negative).

•**Types:**

- **Anionic** (e.g., **SDS – sodium dodecyl sulfate**): negatively charged
- **Cationic** (e.g., **CTAB – cetyltrimethylammonium bromide**): positively charged

🔬 **Properties:**

•**Strongly denaturing** – disrupt protein–protein and protein–lipid interactions by breaking both hydrophobic and electrostatic bonds.

•**Break down membranes effectively**

•Often used for **complete cell lysis** and **protein denaturation** (e.g., in SDS-PAGE)

•Can **disrupt secondary and tertiary protein structures**

⚠️ **Drawbacks:**

•Not suitable if you need to preserve **native protein function** or **antigenicity**

🌊 2. Non-Ionic Detergents

•**Head group: Uncharged**, typically a sugar (e.g., maltoside) or ethylene oxide chain.

•Examples:

- **Triton X-100**
- **NP-40**
- **Tween 20**, **Tween 80**
- **Digitonin**

🔬 **Properties:**

•**Mild** – solubilize membranes without fully denaturing proteins

•Maintain **protein activity and interactions** better

•Often used in:

- **Immunostaining**
- **Immunoprecipitation**
- **Membrane protein extraction**
- **Cell permeabilization** (e.g., Triton X-100 for nuclear staining)

✅ **Benefits:**

•Ideal for applications needing **permeabilization** without **lysis**

•Preserve **antibody binding sites** and **epitope structure**

Types of detergent

Detergent	Type	Typical Concentration	Incubation Time	Notes
Triton X-100	Non-ionic	0.1%–1%	5–15 min	Common for immunostaining, permeabilizes all membranes
Tween-20	Non-ionic	0.05%–0.5%	5–20 min	Milder than Triton X-100, used for surface antigens
Saponin	Natural	0.01%–0.2%	5–30 min	Reversible permeabilization, selective for cholesterol-rich membranes
Digitonin	Natural	0.001%–0.02%	5–15 min	Selectively permeabilizes plasma membrane but leaves organelles intact
NP-40	Non-ionic	0.1%–1%	5–15 min	Stronger than Triton X-100, often used for cell lysis
CHAPS	Zwitterionic	0.1%–1%	10–30 min	Gentle on membrane proteins, good for maintaining protein activity

Key Considerations:

•Higher detergent concentrations or longer incubation times **increase permeability** but can also **damage cellular structures**.

•**Cold incubation (4°C)** minimizes protein denaturation, while room temperature or **37°C** can enhance permeability.

•Some detergents (like **Saponin and Digitonin**) provide **reversible permeabilization**, useful for live-cell studies.

Blocking non specific sites

🎯 What Is Blocking?

Blocking is a preparatory step in immunodetection techniques, used to **prevent non-specific binding** of antibodies to the sample. A **protein- or polymer-based solution** is applied to occupy potential non-specific binding sites **before** the addition of the primary antibody.

🔒 Targets of Blocking Include:

- Non-specific **membrane surfaces**
- **Charged functional groups**
- **Fc receptors**, particularly in immune cells
- **Hydrophobic regions** on proteins or sample supports

🧪 Common Blocking Agents:

- **Bovine Serum Albumin (BSA)**
- **Normal serum** (species-matched)
- **Fish gelatin**

✅ Purpose:

To reduce background signal and **enhance the specificity** and **clarity** of antibody-based detection by ensuring binding occurs **only at target epitopes**.

Blocking non specific sites

Blocking Agent	Commonly Used In	Mechanism
Bovine Serum Albumin (BSA, 1–5%)	Western blot, ELISA, IF	Binds to non-specific protein sites
Non-fat Milk (2–5%)	Western blot	Blocks hydrophobic interactions
Serum (5–10%)	Immunohistochemistry (IHC), IF	Contains proteins to block Fc receptors
Gelatin (0.2–0.5%)	Immunostaining	Forms a protein barrier on surfaces
Casein (0.5–2%)	ELISA, WB	Prevents background from hydrophobic sites

Bovine Serum Albumin (BSA) is a **globular protein** (~66 kDa) derived from cow blood serum.

How BSA Minimizes Non-Specific Binding?

1.Surface Coating 🛡️

1. BSA **binds to exposed surfaces** (e.g., membrane, well plates, slides), preventing antibodies or probes from sticking non-specifically.

2.Protein Saturation 🚫🔗

1. Many **non-specific interactions occur due to empty protein-binding sites** on plastic, membranes, or glass.
2. BSA **fills these sites**, reducing unwanted background signals.

3.Hydrophobic & Electrostatic Interactions ⚡💧

1. Antibodies and detection reagents can **bind non-specifically** due to **hydrophobic** or **ionic** forces.
2. BSA **competes** with these interactions, blocking unintended binding.

Blocking non specific sites

For Immunofluorescence / IHC:

🔹 **1–5% BSA in PBS**

•Dissolve **1–5 g of BSA** in **100 mL PBS**

•**Optional:** Add **5–10% serum** from the same species as the secondary antibody.

•Filter with 0.2 um filter

Key Reason: Blocking Fc Receptor Binding

🛡️ **Immune cells (e.g., macrophages, B cells, dendritic cells) express Fc receptors (FcRs), which can bind antibodies non-specifically.**

🔹 If **secondary antibodies** bind to Fc receptors instead of their intended targets, this leads to **false-positive staining** (high background).

✅ **Solution:**

•If you use a **goat anti-rabbit** secondary antibody, block with **normal goat serum (NGS)**.

•If using a **donkey anti-mouse** secondary, block with **normal donkey serum (NDS)**.

🔬 **How It Works:**

•The **serum contains immunoglobulins (IgG) from that species**, which **saturate Fc receptors** before the secondary antibody is added.

•This **prevents unwanted Fc receptor binding**, reducing **non-specific fluorescence or staining**.

When Is Serum Blocking Important?

✔ **Tissue staining (IHC, IF)** – Some cells express high Fc receptors, increasing background.

✔ **Flow cytometry** – FcR-expressing immune cells (e.g., B cells, monocytes) can trap antibodies.

✔ **Cell culture assays** – Some cell types bind antibodies non-specifically.

Blocking non specific sites

IgG Antibody – Structure & Function 🧪🔬

What is IgG?

•**IgG (Immunoglobulin G)** is the most abundant antibody in the **blood and extracellular fluid**, making up ~75–80% of total serum immunoglobulins.

•It plays a crucial role in **adaptive immunity**, neutralizing pathogens and activating immune responses.

IgG Structure 🏗️

🔷 **Monomeric Y-shaped glycoprotein (~150 kDa)**

🔷 Composed of **four polypeptide chains**:

•2 Heavy Chains (H, ~50 kDa each)

•2 Light Chains (L, ~25 kDa each)

•Disulfide bonds stabilize the structure

Region	Function	Description
Fab (Fragment antigen-binding)	Antigen recognition	Contains variable regions (VH & VL) that bind to specific epitopes
Fc (Fragment crystallizable)	Immune activation	Interacts with Fc receptors (FcγR) on immune cells and activates complement system
Hinge Region	Flexibility	Allows the antibody to bind two antigens at different angles
Constant Region	Effector function	Defines IgG subclasses (IgG1, IgG2, IgG3, IgG4) with distinct immune roles

Common Staining Methods

1. Antibody-based staining

- Primary & secondary antibodies with fluorophores, or labelled primary Abs
- High specificity — targets proteins, structures
- Examples: tubulin, histones, membrane markers

2. Fluorescent chemical compounds

- Small labelled molecules that bind specific cell components cell permeable
- Examples: DAPI / Hoechst → DNA; Phalloidin → actin; MitoTracker → mitochondria

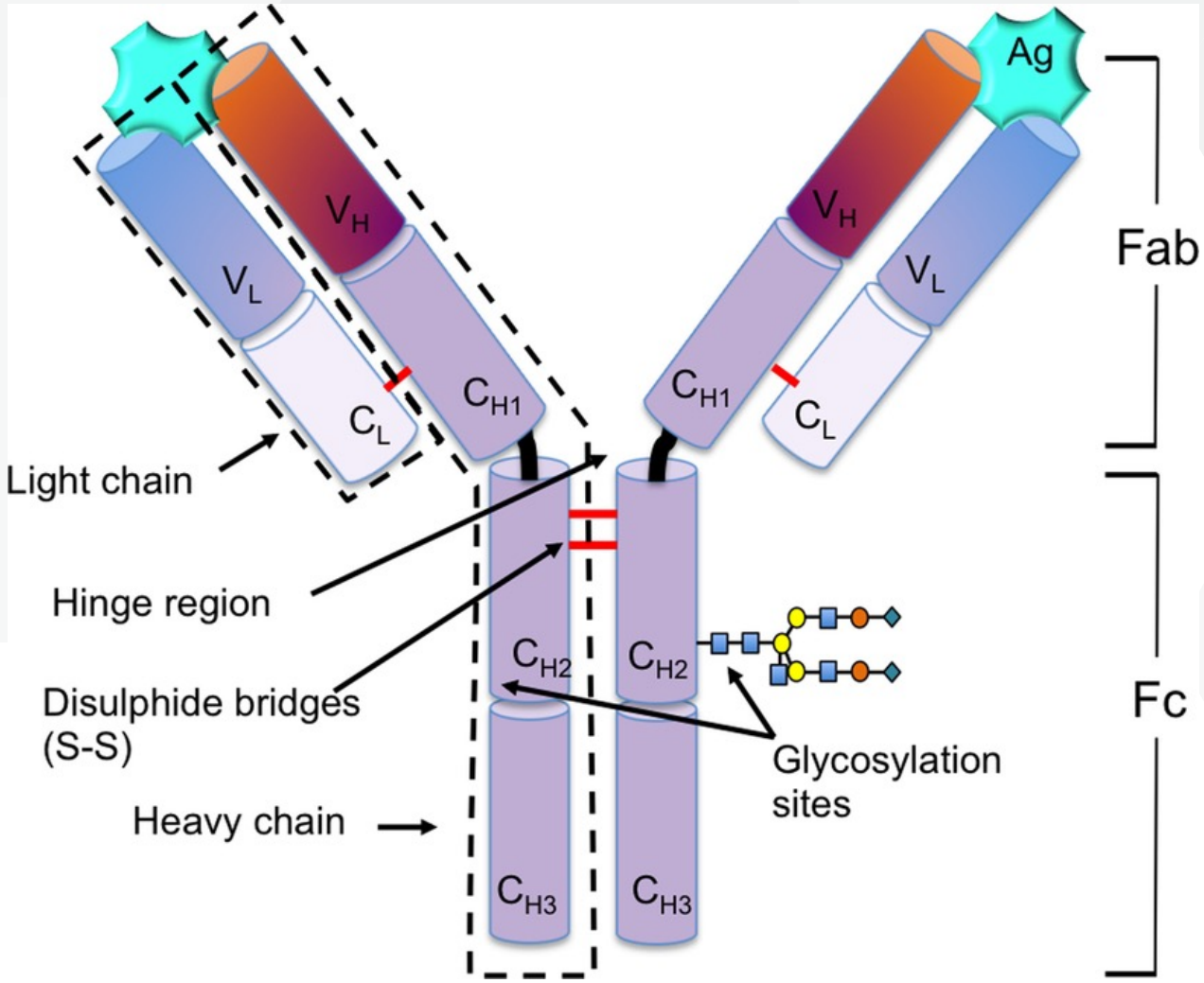
3. Genetically encoded fluorescent tags

- Fluorescent proteins (e.g., GFP, mCherry, mKate, CFP…) fused to target proteins
- Transient or stable expression
- Chromobodies as a subclass (nanobody + FP) – small, cell permeable

4. Fluorescent In Situ Hybridization (FISH)

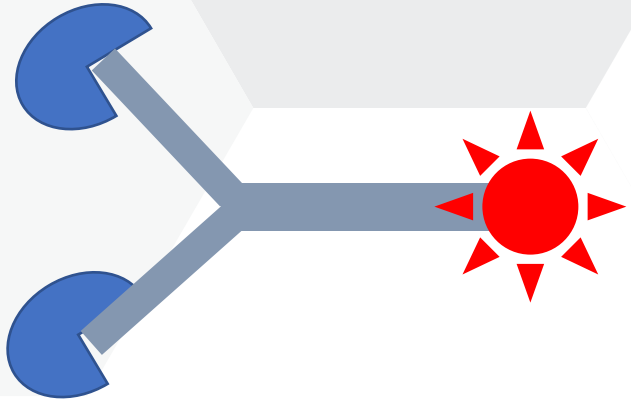
- Uses **fluorescent DNA or RNA probes** to detect **specific nucleic acid sequences**
- Highly sensitive for **mRNA, gene loci, or chromosomes**
- Often used in **diagnostics**, **gene expression analysis**, and **cell identification**

Antibody-based staining



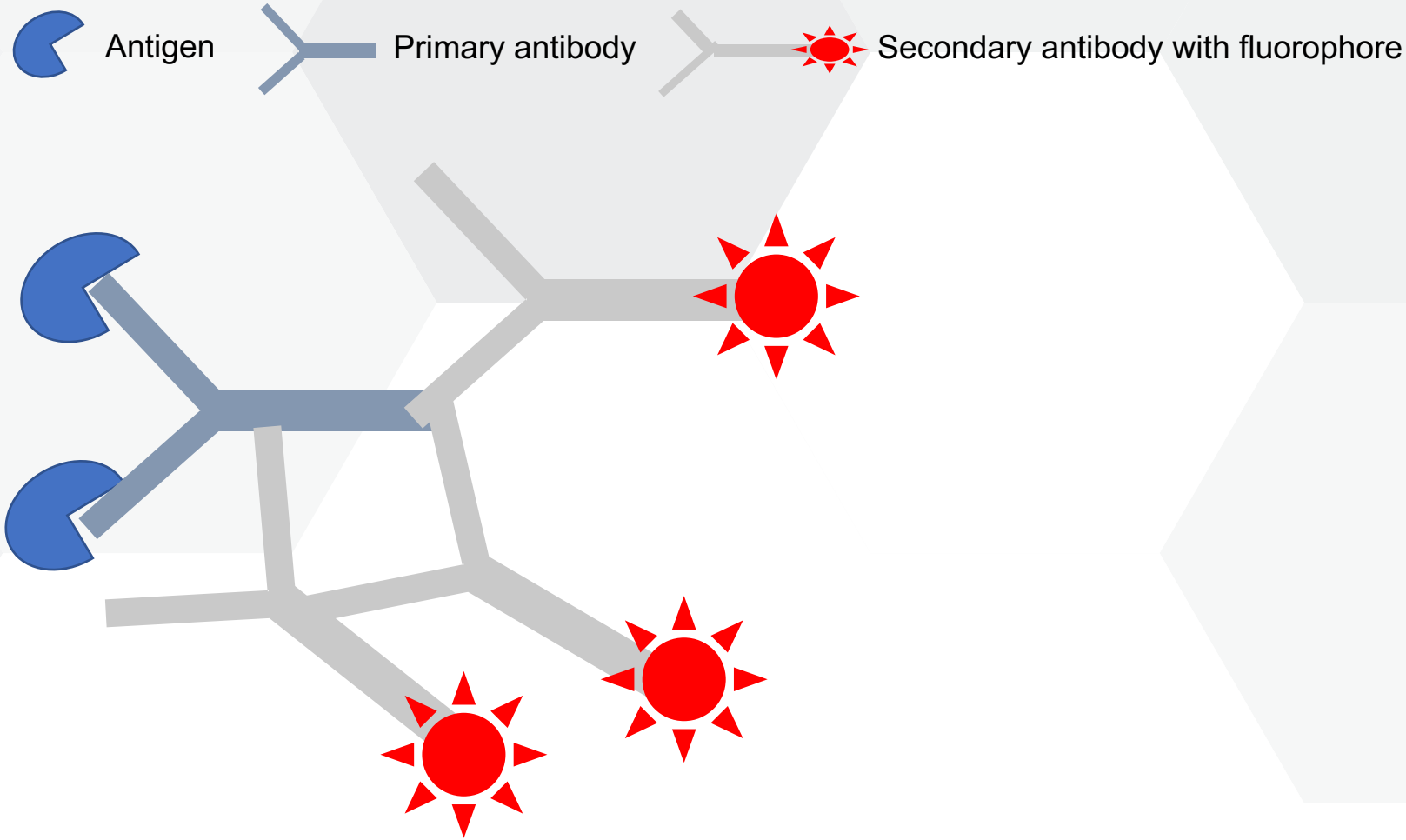
O`Kennedy et al., Antibody Technology Journal, 2016

Direct immunolabelling



Faster, but less sensitive than indirect IF

Indirect immunolabelling



Longer protocol, but more sensitive than direct IF due to signal amplification

Principles of Indirect immunolabelling

•**Primary antibody** binds specifically to the target antigen.
•**Secondary antibody**, conjugated with a fluorophore, binds to the primary antibody.
•Fluorophore emits fluorescence upon excitation, enabling visualization under a fluorescence microscope.

✅ **Advantages**:
•Signal amplification (multiple secondaries per primary).
•Versatile detection using various fluorophores.
•Cost-effective (same secondary used for multiple targets).

Nanobodies

What are Nanobodies?

- **Single-domain antibody fragments** derived from camelid antibodies.
- Much **smaller** than conventional antibodies (~15 kDa vs. ~150 kDa).
- High **binding specificity** and **affinity** to target molecules.

Advantages in Microscopy

✅ **Better tissue penetration** – smaller size allows deeper access.
✅ **Higher labeling density** – more nanobodies can bind per target.
✅ **Minimal background signal** – reduced cross-reactivity.
✅ **Improved photostability** – when conjugated with fluorophores.

Applications in Sample Preparation

🔬 **Immunostaining** – direct labeling with fluorophore-conjugated nanobodies.
🔬 **Super-resolution microscopy** – STED, PALM, dSTORM use nanobodies for higher precision.
🔬 **Live-cell imaging** – minimal perturbation to target proteins.

Nanobodies

Chromobodies in Light Microscopy
What are Chromobodies?
•Genetically encoded **nanoprobes-fluorophore fusion proteins**.
•Derived from **single-domain camelid antibodies (nanobodies)** linked to a **fluorescent protein** (e.g., GFP, mCherry).
•Allow real-time **intracellular labeling of target proteins**.
Advantages in Microscopy
✅ **Live-cell imaging** – monitor protein dynamics in real-time.
✅ **No need for chemical fixation or permeabilization** – avoids sample artifacts.
✅ **High specificity and minimal background** – derived from nanobodies.
✅ **Genetically encoded** – stable expression in cells.
✅ **No permeabilization required** - penetrate cell membrane.
Applications
🔬 **Tracking protein dynamics** – study movement, interactions, and localization.
🔬 **Super-resolution microscopy** – useful for STED and PALM techniques.
🔬 **Functional studies** – chromobodies can be fused to reporters for advanced analyses.
🔬 **Cell division and cytoskeleton studies** – visualize actin, tubulin, and mitotic processes.

Fluorescent chemical compounds

🔷 **Definition**:
- Small synthetic or natural molecules that bind to specific cellular components
- Provide strong fluorescence signals without genetic modification

🔷 **Key Features**:
- Fast and easy to apply
- Often cell-permeable
- Available in various spectral properties (colors, photostability, pH sensitivity)
- Should not alter other processes or cell viability – not toxic

🔷 **Examples & Targets**:
DAPI / Hoechst → DNA (nucleus)
Phalloidin → F-actin (cytoskeleton)
MitoTracker → mitochondria
LysoTracker → lysosomes
FM dyes → plasma membrane & endocytosis tracking

✅ Widely used in fixed and live-cell imaging to visualize organelles, structures, and processes.

Fluorescent proteins

What are Fluorescent Proteins (FPs)?

🔹 Genetically encoded fluorophores
🔹 Fused to a protein of interest → real-time localization
🔹 Expressed in live cells via transfection or stable integration

Common FPs:

•**GFP** (Green Fluorescent Protein)
•**mCherry**, **RFP**, **tdTomato** (Red variants)
•**mTurquoise**, **CFP** (Cyan)
•**YFP**, **Venus** (Yellow)

Advantages:

✅ Live-cell compatible
✅ No staining needed
✅ Track dynamic processes (e.g. mitosis, transport)
⚠️ Considerations:

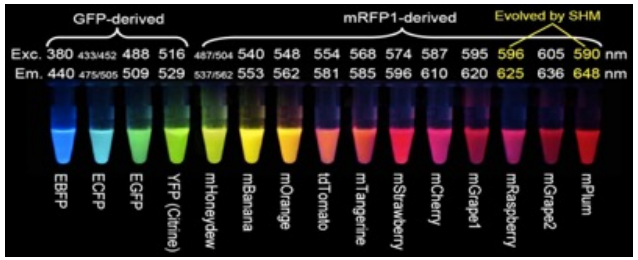
•Overexpression artifacts
•Photobleaching
•Requires appropriate filters & stable expression system

Fluorescent proteins

- 🔷 **Origin**:
- - First discovered in the jellyfish *Aequorea victoria*
- - Green Fluorescent Protein (GFP) isolated in 1960s, gene cloned in 1992
- 🔷 **Historical Milestones**:
- - 1994: GFP expressed in other organisms → revolution in live-cell imaging
- - 2008: Nobel Prize awarded to Shimomura, Chalfie, and Tsien for GFP
- - Development of color variants: CFP, YFP, mCherry, tdTomato, etc.
- 🔷 **Current Use**:
- - Fused to proteins of interest to monitor localization and dynamics
- - Used in live-cell imaging, biosensors, FRET, photoconversion, and optogenetics
- - Available in multiple colors, photoactivatable or pH-sensitive forms
- ✅ Fluorescent proteins allow real-time, non-invasive visualization of proteins and processes in living cells.

Genetically encoded fluorescent tags

Protein	Color	Excitation / Emission (nm)	pK_a	Brightness	Photostability	Lifetime (ns)	Maturation Time (h)	Key Features
GFP	Green	488 / 509	5.5	~33	High	2.6–2.8	~0.5–1.0	Benchmark FP; robust & versatile
mNeonGreen	Bright Green	506 / 517	5.7	**~90**	High	~3.9	~0.5	Extremely bright, fast maturing
YFP	Yellow	514 / 527	6.9	~45	Medium	~3.2	~0.7–1.0	FRET donor; pH-sensitive
CFP	Cyan	433 / 475	4.7	~17	Low	~2.3	~1.0	FRET donor; low brightness
mTagBFP2	Blue	399 / 456	2.7	~30	High	~2.7	~0.6	Bright and photostable in blue
mCherry	Red	587 / 610	4.5	~16	High	~1.5	~1.5	Stable and monomeric; slower maturation
tdTomato	Orange-Red	554 / 581	4.7	**~95**	Medium	~3.1	~1.0	Extremely bright, dimeric
mKate2	Far-Red	588 / 633	5.4	~34	High	~3.0	~0.7	Far-red; great for deep tissue imaging



Fluorochromes

https://www.thermofisher.com/cz/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html

Alexa dyes
CF dyes
FITC
Rhodamine
Cy3
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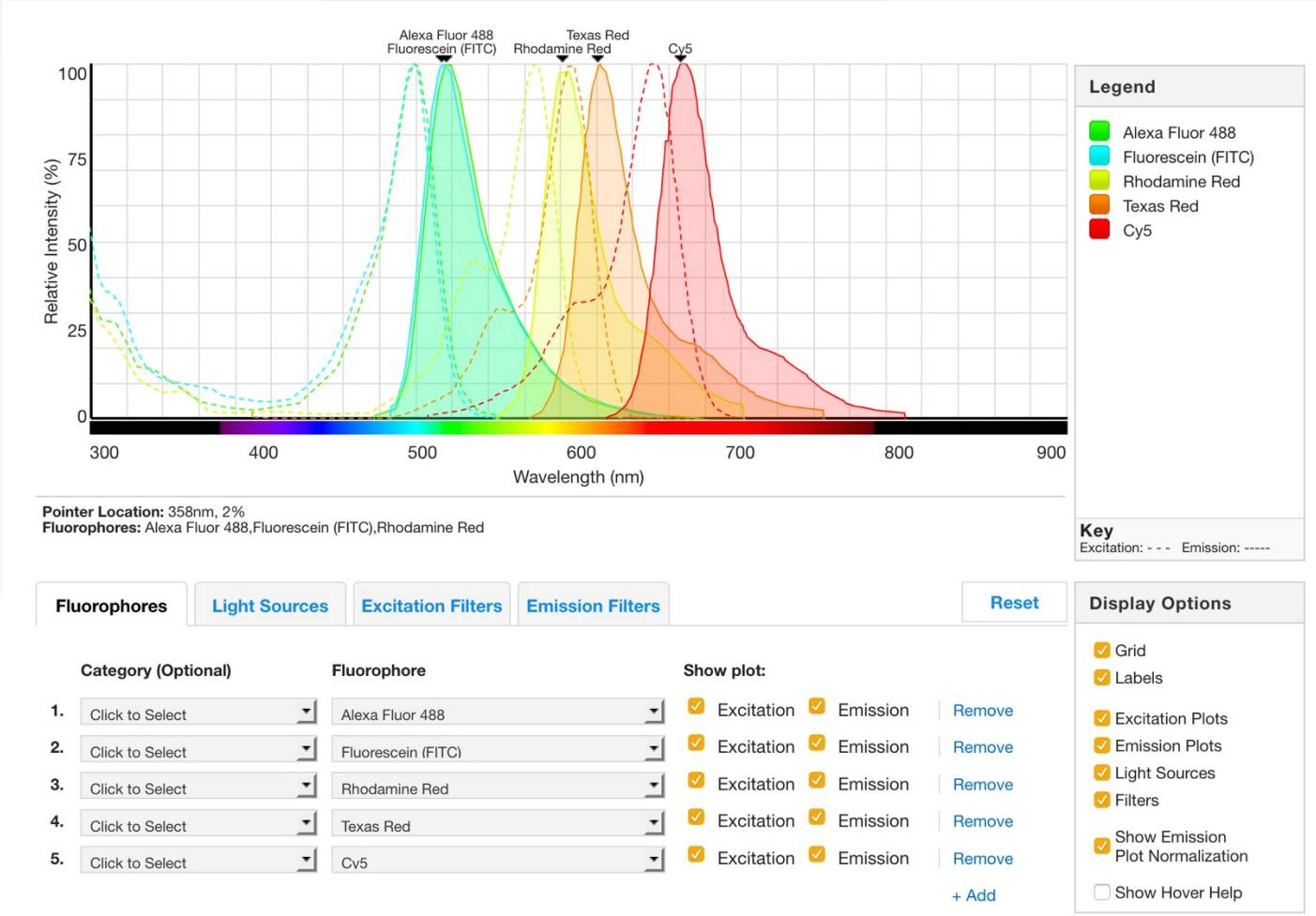

Check he excitation and detection capabilities of the selected microscopy system

Photostability and Fluorophore Protection

What is Photobleaching?
•Loss of fluorescence due to light-induced chemical damage
•Irreversible — leads to fading signal during imaging

Factors affecting photostability:
- 🔹 Intensity & duration of excitation light
- 🔹 Fluorophore type (e.g., Alexa Fluor vs. FITC)
- 🔹 Mounting medium and oxygen levels

How to improve photostability:
- ✅ Use **antifade reagents** (e.g., ProLong Gold, Vectashield)
- ✅ Limit exposure time and excitation intensity
- ✅ Use **photostable dyes** (e.g., Alexa Fluor series)
- ✅ Use fast acquisition modes when possible

Mounting media and sample preparation

Purpose of Mounting

- Preserves sample for imaging
- Protects against drying and photobleaching
- Refractive index matching for microscopy

Types of Mounting Media

✅ **Aqueous media**

•Suitable for water-based stains and live imaging
•Example: PBS + glycerol

✅ **Hard-setting (curing)**

•Long-term storage, coverslip sealing
•Contains antifade reagents to reduce photobleaching
•Example: ProLong Gold, Fluoromount-G

Enable long-term storing of samples

Indirect immunolabelling protocol example

Step-by-Step Protocol

1. **Fixation**:
   - 4% paraformaldehyde in PBS, 10–15 min at RT
2. **Permeabilization**:
   - 0.1% Triton X-100 in PBS, 5–10 min
3. **Blocking**:
   - 1–5% BSA or serum in PBS, 30-60 min, at RT
4. **Primary Antibody Incubation**:
   - Dilute in blocking buffer, 1 h at RT or overnight at 4 °C
5. **Wash**:
   - 3× with PBS, 5 min each
6. **Secondary Antibody Incubation**:
   - Fluorophore-conjugated, 1 h at RT
7. **Counterstain (optional)**:
   - e.g., DAPI for nuclei
8. **Mounting**:
   - Antifade medium, seal coverslip

🔬 Ready for fluorescence microscopy

Live cell staining protocol example

Live Imaging – SiR-actin Staining

1️⃣ **Prepare staining solution**:

•Dilute **SiR-actin** (recommended 0.5–1 µM)

•Use phenol red–free, serum-free medium if possible

2️⃣ **Optional: Add Verapamil**

•10 µM to inhibit efflux pumps (improves staining)

3️⃣ **Incubate cells**:

•30–60 min at 37 °C, 5% CO_2

4️⃣ **Image directly (no wash)**:

•Live-cell confocal or spinning-disk microscope

✅ SiR-actin is fluorogenic — emits in far-red (exc. 640 nm / em. 661 nm)

✅ Compatible with long-term live-cell imaging

Live cell staining protocol example
Dual staining

Live Imaging – Hoechst + SiR-Actin

1️⃣ **Prepare staining solution**:

- **Hoechst 33342**: 1 µg/mL
- **SiR-actin** (Spirochrome): 0.5–1 µM
- Optional: **Verapamil** 10 µM (improves SiR-actin retention)
- Use phenol red–free, serum-free medium

2️⃣ **Incubate cells**:

- 30–60 min at 37 °C, 5% CO_2

3️⃣ **Image live**:

- Hoechst → DAPI channel (exc. 350 nm / em. 461 nm)
- SiR-actin → far-red channel (exc. 640 nm / em. 661 nm)

✅ No fixation or permeabilization needed

✅ Compatible with dynamic, time-lapse imaging

✅ Excellent signal-to-noise and minimal background

Near infrared (NIR) fluorochromes

What are NIR Fluorochromes?

- 🔹 Fluorophores excited and emitting in the **700–900 nm** range
- 🔹 Minimal autofluorescence and low light scattering
- 🔹 Ideal for **deep tissue imaging** and **high-contrast labeling**

Examples:

- **SiR dyes** (e.g., SiR-DNA, SiR-actin)
- **IRDye 800CW**, **Alexa Fluor 750**
- **Cy7**, **DyLight 800**

Advantages:

- ✅ Low background fluorescence
- ✅ Penetrates deeper into tissues
- ✅ Less phototoxicity — ideal for live imaging

⚠️ Considerations:

- Requires specialized filter sets or detectors
- Lower quantum yield than visible-range dyes
- May be less bright on conventional microscopes

Practical part I.
Visualize nucleus and tubulin-actin cytoskeleton

Fix and stain cellular structures using an anti-Tubulin antibody, Hoechst, and Phalloidin to visualize the nucleus and tubulin-actin cytoskeleton. Subsequently, measure the size and morphology of the nuclei and cytoplasm.

Cells:
Mouse Embryonic Fibroblasts

Reagents:
Hoest 33342 (ThermoFisher, Cat. 62249)
PBS
Triton-X100 (Merck, Cat. T8787)
Tween 20 (Merck, Cat. P9416)
Bovine Serum Albumine (Merck, Cat. A7030)
Anti alpha tubulin, mouse monoclonal (Merck, Cat. T7451)
Anti mouse IgG - Alexa 488 (Thermo, Cat. A11001)
Phalloidin-Alexa647 - (Merck, Cat. 65906)

Practical part I.
Visualize nucleus and tubulin-actin cytoskeleton

Step-by-Step Protocol - ultra fast protocol

1. **Fixation:** 4% paraformaldehyde in PBS, 10 min at RT
2. **Wash:** 3× with PBS, 2 min each
3. **Permeabilization:** 0.5% TritonX100 in PBS, 10 min at RT
4. **Blocking:** 3%BSA in PBS, 15 min at RT
5. **Primary Ab:** Add primary Ab, 20 min at RT
6. **Wash:** 3× with PBS, 2 min each
7. **Secondary Ab:** Add secondary Ab, 1:1000 dilution,10 ug/ml of Hoechst, Phalloidin 1:500, 20 min at RT
8. **Wash:** 3× with PBS, 2 min each
9. **Mount**: remove PBS and add drop of Vectashield (optinally you can add coverslip on top)

🔬 Ready for fluorescence microscopy

Practical part I.
Visualize nucleus and actin cytoskeleton in fixed cells

Acquire at least 10 cells

1) measure area, length axis X,Y of nuclei. Calculate mean value.

2) measure thickness of actin and tubulin fibres. Calculate mean value.

Practical part II.

Stain cells with Hoechst and sir-Act dye in order to visualize nucleus and actin cytoskeleton without fixation in live cells.
Measure size and morphology of nuclei and cytoplasm in living cells.

Cells: Mouse Embryonic Fibroblasts
Dyes: Hoechst (ThermoFisher, Cat. 62249), sir-Act dye (Spirochem, Cat. SC006)

Step-by-Step Protocol

1. **Stain**: 10 ug/ml of Hoechst 33342 and sir-Act (1:1000) in cell culture media, 30 min at RT
2. Optional (Wash and add fresh media)

🔬 Ready for fluorescence microscopy

Practical part II.
Visualize nucleus and actin cytoskeleton in living cells

Acquire at least 10 cells

1) measure area, length axis X,Y of nuclei. Calculate mean value.

2) measure thickness of actin fibres. Calculate mean value.