Immunostaining Protocols

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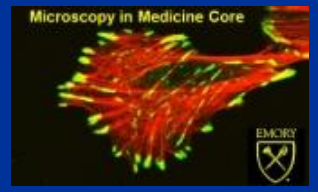

http://medicine.emory.edu/MIMCore

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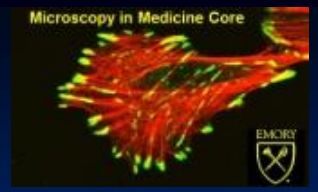
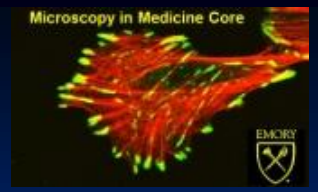


Variables in "standard" immunostaining protocol

2-step or indirect immunofluorescence

1. **Substrate on which cells are plated**: plastic vs. glass; coating vs. non
2. **Plating density**: sparse vs. confluent
3. **Time in culture**
4. **Fixation**: solvent vs. aldehyde
5. **Permeabilization**: detergents (Triton X-100, NP-40, Tween 20)
6. **Quenching** of aldehydes with NH_4Cl
7. **Blocking** sites prone to nonspecific interactions: PBS + BSA
8. **Choice of primary Ab**
9. **Choice of secondary Ab**
10. **Mounting medium** for microscopic examination



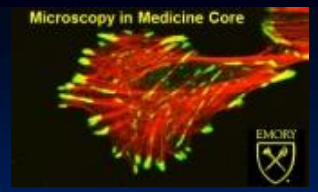
1. Substrate on which cells are plated: plastic vs. glass

Plastic dishes/coverslips are NOT optimal for high resolution imaging

Therefore, GLASS coverslips (#1.5) should be used whenever possible

CLEANLINESS IS NEXT TO GODLINESS in microscopy

- SO MAKE SURE **EVERYTHING** IS CLEAN
- FILTER FIXATIVE AND BLOCKING SOLUTIONS TO REMOVE ANY PRECIPITATES
- WIPE OFF GLASS SLIDES AND COVERSLIPS
- STERILIZE COVERSLIPS
- DIP IN ALCOHOL AND FLAME OR AUTOCLAVE coverslips to sterilize



Coating slides and coverslips

Gelatin (Human Umbilical Endothelial Cells or HUVECs)

- Dissolve gelatin in H_2O
- Autoclave to sterilize
- Pipette gelatin onto coverslips and leave 15-30 min RT
- Aspirate gelatin and add media containing cells

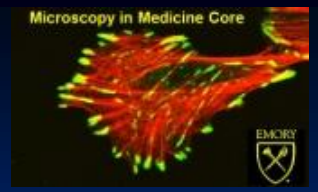
Poly-L-lysine

- Prepare 500 µg/ml poly-L-lysine (MW> 150K; Sigma P 1399) in H_2O
- Coat slides or coverslips
- Incubate 10 min RT
- Rinse 3X sterile H_2O
- Air dry

ECM components

- Collagen
- Laminin
- Fibronectin

Matrigel



2. Plating density: sparse vs. confluent

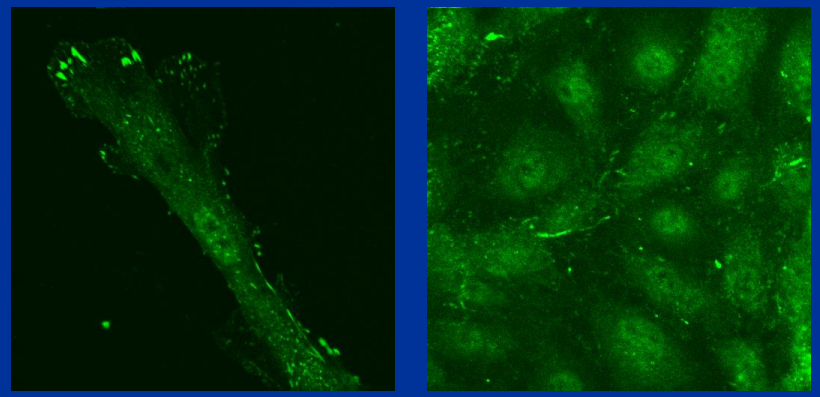
Plating density depends upon application, but the goal is to obtain an **even distribution of cells, separated by a workable distance**

EXAMPLE: Plating RASMs on 22 mm diameter coverslips in 6-well dish

Trypsinize rat aortic smooth muscle cells (RASMs) and count with hemocytometer. Dilute cells to final concentration of 2 X 10^4 cells/ml. Add 4 mls to each multiwell (2 X 10^4 cell/ml X 4 ml= 8 X 10^4 cells/multiwell).

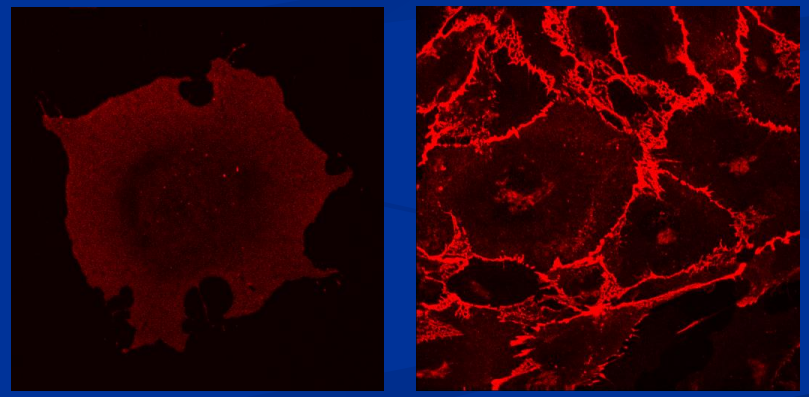
Endothelial cells such as HUVECs can be plated either sparse or confluent

p22phox in focal adhesions in single cells; hard to see in confluent cells



Sparse Confluent

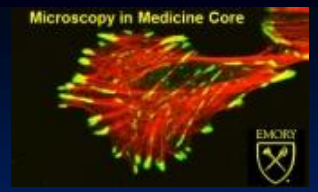
VE cadherin in cell-cell junctions only in confluent cells



Sparse Confluent

3. Time in culture

Time to reach confluency or time to replenish surface receptors disrupted by trypsinization



4. FIXATION (Chemical)

Before fixing, OBSERVE cells with phase contrast or DIC to assess healthy cells

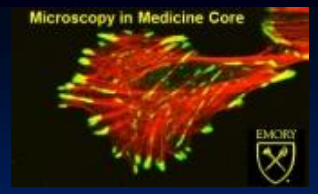
A. Solvent: Coagulating fixatives coagulate or extract proteins
Major problem is shrinkage

B. Aldehydes: Covalently crosslink proteins
Do not crosslink lipids

The aim of **FIXATION** is to balance the preservation of structural integrity with preservation of antigenic sites

Types of fixatives:	Structure	Antigen preservation
A. SOLVENT FIXATION		
➢ Methanol (100%) (-20° C 10 min)	Excellent	Sometimes poor (May need detergent permeabilization)
➢ Acetone (-20° C 10 min)	Poor	Usually excellent
➢ Combination of methanol and acetone (50%/50% or 90% acetone/10% methanol)	Good compromise	
➢ 95% Ethanol	Good	Good

NEVER use acetone in plastic dishes

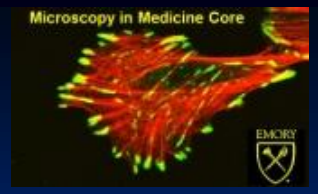


B. ALDEHYDE FIXATION: CAUTION: USE FUME HOOD AND WEAR GLOVES

Dispose of waste aldehydes to Chemical Safety

	Structure	Antigen preservation
➢ Formaldehyde	Excellent	Poor if contains methanol Use methanol-free preparations from Polysciences (#04018)
➢ **Paraformaldehyde (4% made from powder by heating and addition of NaOH)** or use Polysciences (#00380) already in solution	**Excellent**	**Excellent**
➢ Glutaraldehyde	Superior	Poor Used mostly in electron microscopy; most antigen binding sites destroyed and lots of autofluorescence

Best fixation scheme is determined **empirically**



5. Permeabilization agents

Detergents may be used to allow access of Ab molecules into cells (increase access to sites located beneath plasma membrane by solubilizing or extracting lipids from membrane)

Common detergents dissolved in preferred buffer (usually PBS)

- **Triton X-100** (e.g., 0.05-0.5% Triton for 5-10 min)
- **NP-40**
- **Tween 20**

Concentrations and times of exposure have to be determined.

6. Quenching of aldehydes to reduce background

Unreacted aldehydes in solution are quenched with **NH_4Cl.**

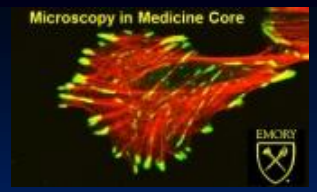
7. Blocking to reduce the background labeling

DO NOT OMIT THIS STEP

Common blocking agents:

- **Bovine serum albumin (BSA)**
- **Normal serum** from secondary antibody source (e.g., normal goat serum)

Concentrations and times of exposure have to be determined.



8. Choice of primary antibody

Antibodies recognize and bind to specific "epitopes." They may be either

Monoclonal: recognizes single epitope

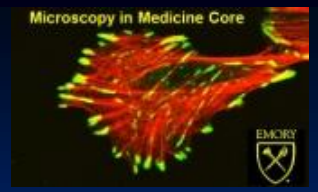
Polyclonal: a collective of antibodies recognizing different epitopes of same molecule

Start by checking **published literature** (e.g., peer-reviewed Cell Biology journals) to see what antibody and processing protocols others have used **SUCCESSFULLY**

Read CAREFULLY **specification sheet** that came with Ab from vendor to determine storage conditions, dilution, incubation recommendations, sequence used to generate Ab, species of antigenic peptide, species of Ab, Ig isotype

Variables that affect binding:

- **Concentration**
  - Perform serial dilutions: use recommended dilution as **STARTING POINT**
- **Incubation time**
  - Check literature or spec sheet
- **Incubation temperature**
  - Room temperature vs. cold
    - If incubation in cold, perform **OVERNIGHT** and do **NOT** let specimens dry (put in humidified chamber)



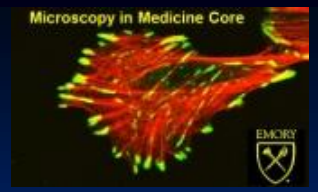
Check antibody specificity and quality by **immunoblotting or immunoprecipitation**

- Immunoblot should show specific bands and NOT zillions of bands
- **MAJOR DIFFERENCE** between immunostaining and immunoblotting is that proteins are fully denatured for immunoblots, whereas in immuno-staining many of the proteins retain a much more native state. **THEREFORE**, Ab that works well in immunoblots may NOT work for immunostaining and vice versa.

Controls for specificity:

- Replace primary Ab with similarly diluted normal serum from same species
- Omit primary Ab
- Use positive controls (different cells) that contain protein
- Use several different antibodies that are directed against same protein
- Preabsorption controls; mixing Ab with protein or antigenic peptide before labeling to eliminate binding; also preincubating cells with antigenic peptide
- Use antigen-negative cells (cells from knock-out mouse)
- Use GFP fusion proteins
- In double labeling experiments, **ALWAYS** perform single label controls

Caveat: To produce superior localization without artifact, a GOOD antibody is ESSENTIAL



9. Choice of secondary antibody

Immunologically, the primary Ab dictates the secondary Ab required.
Secondary must be targeted against appropriate class of Igs

e.g., if mouse monoclonal primary Ab, use goat anti-mouse IgG

When double labeling, primary Abs should be from different species and fluorochromes must contrast.

Choice of fluorochromes:

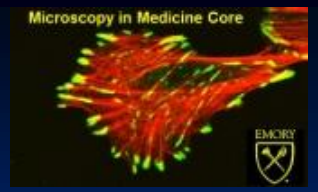
FIND OUT WHAT FILTER SETS, LASER OPTIONS are available to you **BEFORE** ordering a fluorochrome-labeled secondary Ab

Vendors for secondary Abs

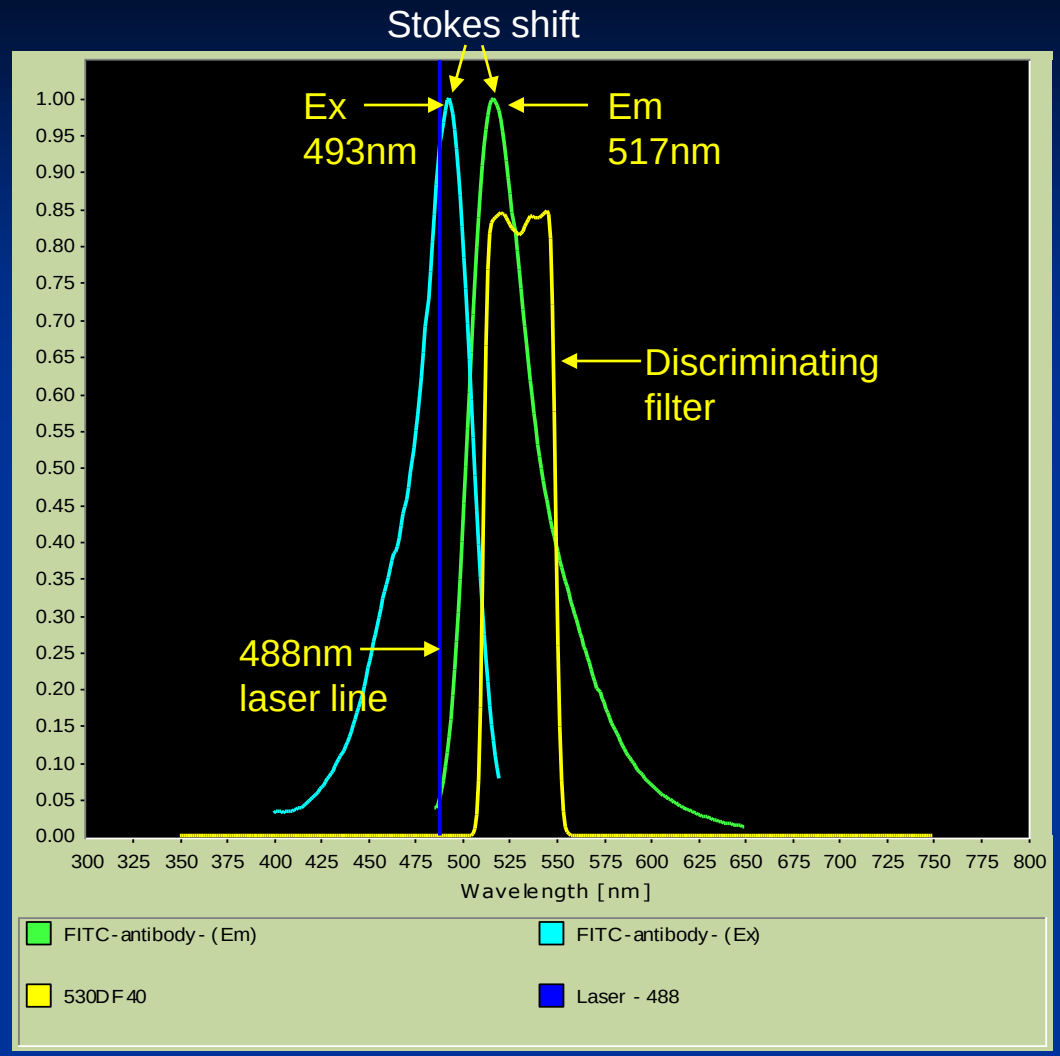
Jackson ImmunoResearch
Alexa dyes from Molecular Probes
Cy series of dyes from Jackson or from Amersham Pharmacia

Application options:

1. Primary X, secondary X, then primary Y, secondary Y
2. Primaries X and Y, then secondaries X and Y



Excitation and emission spectra of FITC (fluorescein) Ex/Em=493/517 nm



488 nm line of argon laser excites near peak maximum of Ex:493 nm

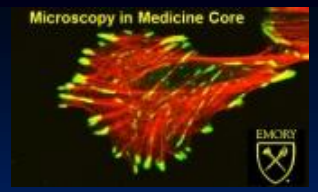
Discriminating filter with narrow band of 40 nm: good for dual labeling BUT results in loss of signal

Fluorescein is excellent fluorochrome:

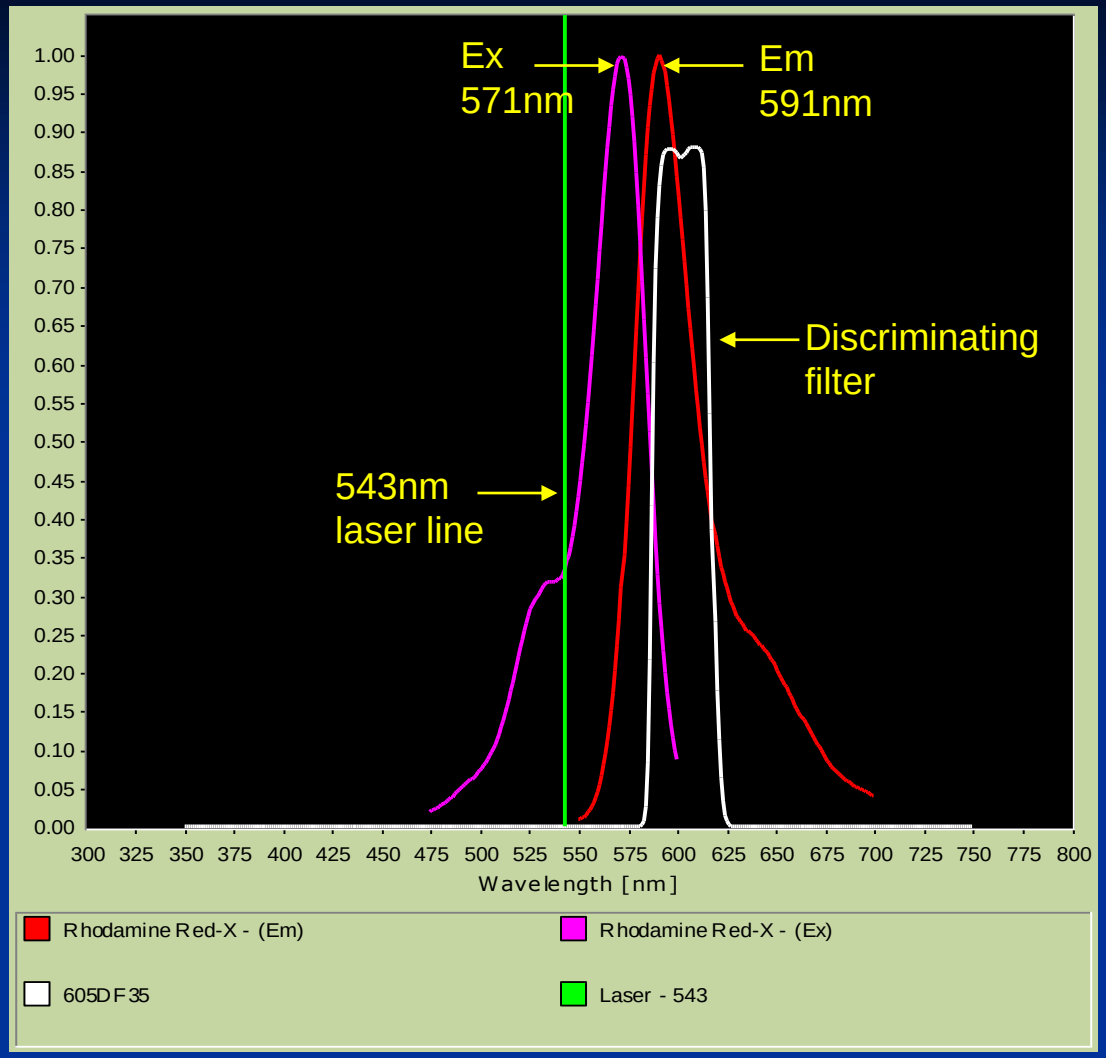
- high probability of absorbing photon (high extinction coefficient)
- high probability of emitting photon (high fluorescence quantum yield)
- Stokes shift=30 nm

Disadvantages of **fluorescein**:

- Rapid photobleaching (irreversible photochemical destruction of dye)
- pH sensitive



Excitation and emission spectra of Rhodamine Red X Ex/Em=571/591 nm



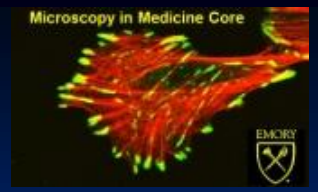
543 nm line of argon laser does not excite near excitation maximum, but still excites molecule

RRX:

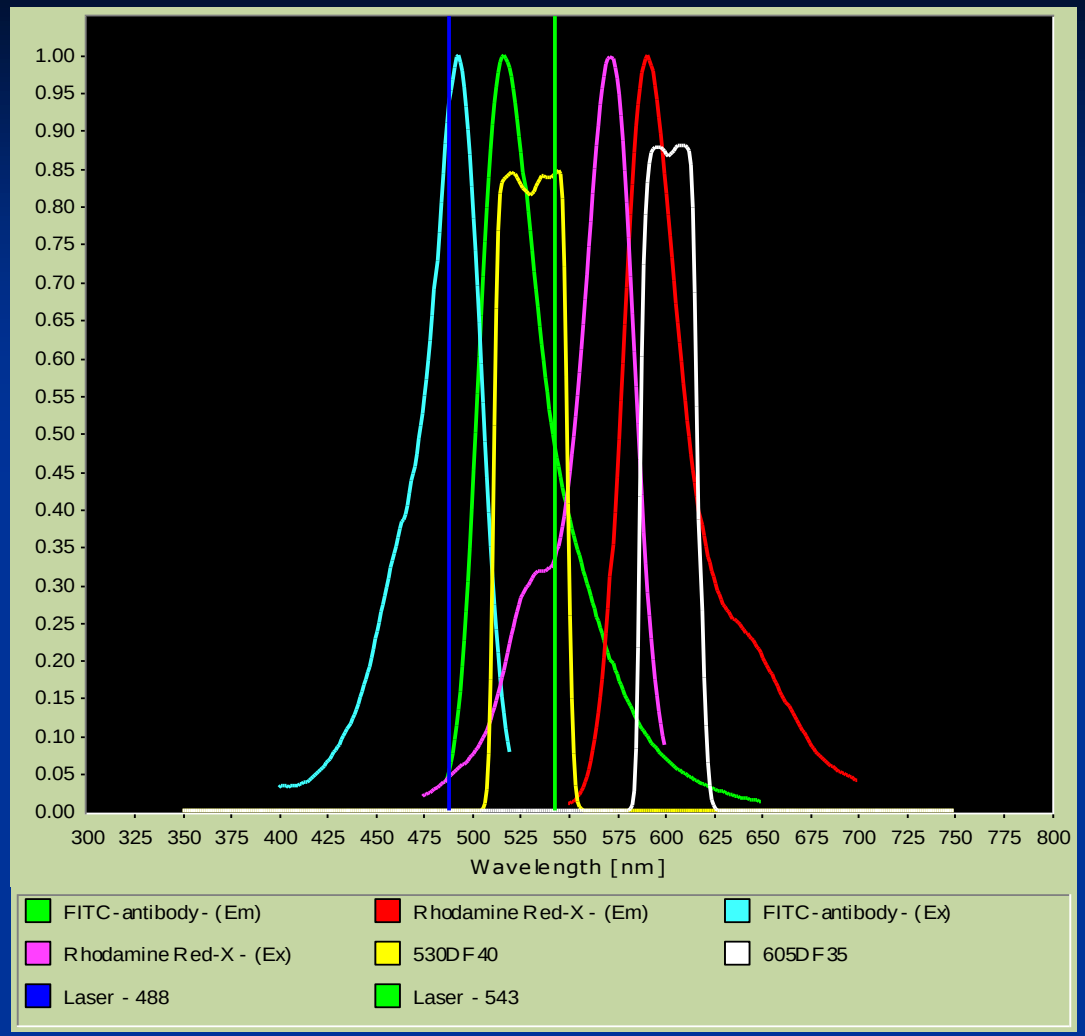
- More photostable than fluorescein
- Not pH sensitive

Alexa Fluor dyes (Molecular Probes) may be preferable to **FITC** or **Rhodamine**:

- Brighter
- More photostable
- Not pH sensitive
- Narrow emission spectra



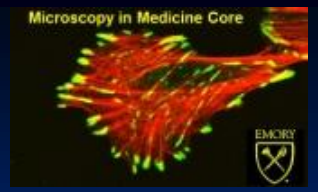
Dual labeling with fluorescein and RRX



Simultaneous imaging may result in crosstalk (bleed-through) where "green channel" detects signal from red label and "red channel" detects signal from green label

One solution:

Collect individual channels separately (**sequentially**), then merge images in software or Photoshop



10. Mounting medium

Choose a mounting medium that has the same refractive index as the immersion liquid of the lens you are going to use

Avoid refractive index mismatch: do not use oil lens with specimen mounted in aqueous media

For fluorescence, select a mounting medium containing an anti-oxidant (anti-fade agents scavenge free radicals liberated by excitation of fluorochromes)

Common mounting media	RI
➢ Vectashield®, Vector Labs	1.4577
➢ MOWIOL® Can make own	
➢ Slow-fade® Molecular Probes	

Reduce air bubbles. **Mounting a coverslip is more of an art than science.**

Remove excess media (blot or aspirate)

NEVER PUT DIRTY SLIDE ON MICROSCOPE STAGE to come into contact with the microscope lenses

Seal the edges of the coverslip with wax or nail polish (Wet ‘n Wild Clear Nail Protector®)