Sample preparation for fluorescence microscopy

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Cells are transparent

HeLa cells by DIC
neutrophil is "chasing" *Staphylococcus aureus*

http://www.microscopyu.com/staticgallery/diphasecontrast/heladic.html

David Rogers, Vanderbilt University
Imaging of proteins and organelles by Fluorescence microscopy methods

- To understand the function of proteins, it is essential to know their localization in cells
- Similarly, to understand the function of organelles, it essential to visualize them

Outline

- Ways on labeling proteins and other cellular structures
  - Immunofluorescence microscopy – specific antibodies
  - Green Fluorescent Fusion (GFP) –proteins
    - transfection of DNA constructs
  - Small fluorescently labeled chemical compounds, which bind with a high specificity to molecules of interest
- Choosing the fluorophore – which color?
1. Immunofluorescence microscopy

- Requires specific antibodies (primary antibody) that recognize protein of interest
- The primary antibody can be directly coupled to a fluorophore, or more commonly a fluorescent secondary antibody that recognizes the primary antibody is used

+ reveals the localization of the endogenous (cell's own) protein
- finding a good antibody can be difficult
- does not permit live-cell imaging

Simultaneous visualization of two proteins by immunofluorescence microscopy

- two primary antibodies raised in different species (e.g. mouse and rabbit) are used.
  Each antibody recognizes only one of the proteins of interest
- two species-specific secondary antibodies are used (e.g. anti-mouse and anti-rabbit).
  These secondary antibodies are conjugated with different fluorophores.
Critical steps in immunofluorescence protocol

- Grow cells on coverslips
  - thickness! #1.5 → 0.17 mm

- Fixation
  - cross-linking - aldehydes
  - (precipitation – organic solvents)

- Permeabilization (if using cross-linking)

- Incubation with the antibodies (optimized dilutions)

- Mounting (remember refractive index!)

Mounting media

- Preservation of the sample
- creating conditions to permit as good imaging as possible

- Matching of the immersion medium and mountant
  - mismatch will cause image degradation due to spherical aberrations and signal loss
  - Think in advance, which objective you will/can use

  Refractive index

<table>
<thead>
<tr>
<th>Sample</th>
<th>Refractive Index</th>
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<tbody>
<tr>
<td>Cells</td>
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<td>Glass</td>
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<tr>
<td>Oil</td>
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</table>

The mountant and the immersion medium should be matched within 0.01-0.05
Mounting media

- Base:
  - aqueous
  - oil
  - plastic

- Antifade
  - Most are reactive oxygen scavengers
  - DABCO
  - n-Phenylenediamine (PPD)
  - n-Propyl gallate (NPG)

- Hardening and non-hardening mountants
  - If using a mountant that does not harden, need to seal the coverslip to prevent drying and may also need spacers to prevent squashing of the specimen

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In general about sample preparation...

- After optimizing the protocol, maintain constant sample preparation
  
- If using cells, keep to good cell culture practices
  - healthy cells

- Standardize everything

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[Table of Mounting Media]

<table>
<thead>
<tr>
<th>Mountant (Click for product link)</th>
<th>Manufacturer</th>
<th>Supplier</th>
<th>Base</th>
<th>mL</th>
<th>Cost/500</th>
<th>Purchased volume (mL)</th>
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www.uhnresearch.ca/wcif
2. **Green Fluorescent Protein (GFP) –fusion proteins**

- GFP is a fluorescent protein
- The protein of interest can be linked to GFP (either to its N- or C-terminus) to make a fusion protein
- Also other spectral variants (different colours) are nowadays available, allowing simultaneous visualization of several proteins in the same cell
- + No need for an antibody, imaging in both fixed and live cells possible → dynamic studies
- - Overexpression of the protein of interest, sometimes GFP-fusion impairs protein function

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**Cloning GFP-fusion proteins**

- Clone the cDNA of the protein of your interest in frame with the GFP in either (or both) vectors
- Transfect the DNA construct into cells
Studying the sub-cellular localization of a protein by using GFP-fusion proteins

1. Cells are transfected with a plasmid that contains the DNA for production of the fusion protein (GFP-protein X).
2. Cell produces a fluorescent fusion protein that localizes similarly to the endogenous (cell's own) protein X.

Endogenous protein X

GFP-protein X – fusion protein.

If fixing cells, use coverslips as for immunofluorescence.

If doing live-cell imaging, use either Mattek-dishes (inverted microscope), or normal cell culture dishes of suitable size (upright microscope).

Examples of live cell imaging with GFP-fusion proteins

Melanoma cell expressing GFP-actin

Fibroblasts expressing MAL-GFP and stimulated with serum
**Things to consider when using GFP-fusions**

- Test the functionality of the fusion proteins
  - compare the localization to endogenous proteins
- Try the fusion protein on both ends of your protein
- Remember: you always overexpress the protein
  - try to use as low expression levels as possible

![Endogenous MAL, MAL-GFP, GFP-MAL](image)

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**3. Small Fluorescent Compounds**

- phalloidin, which is a toxin isolated from mushrooms, binds actin filaments with high specificity

![Alexa 594 Phalloidin](image)

- DAPI (4′,6-diamidino-2-phenylindole) is a fluorescent stain that binds strongly to A-T rich regions in DNA

+ high specificity, labels endogenous protein/structure
- Compounds available to a limited number of proteins, often not suitable for live-cell imaging (because may impair function of the protein/structure)

![Alexa 594 Phalloidin DAPI](image)
Which color???

Choosing the right fluorophore

- Depends on the properties of the microscope you intend to use
  - excitation \rightarrow what can you excite \rightarrow light source
  - emission \rightarrow what can you detect \rightarrow filters
    (if need several colors especially important!)

→ When planning the experiment, find out the properties of your microscope!!!
**Multicolour imaging**

- Often several proteins/structures need to be visualized simultaneously - co-localization
- Need to be sure that can get a specific signal from each colour!

Controls for Multicolour imaging

- Sample that has not been stained at all → to learn to recognize autofluorescence from the cell
- If using antibodies, staining with secondary antibody only → to learn to recognize specific antibody staining
- Samples with the different colours separately → to set up the imaging conditions such that there is minimal cross-excitation and channel bleed-through → to get an estimate of the crosstalk, which can be used to correct images post-acquisition
- If studying co-localization → quantify for example with Pearson`s coefficient
Fluorophore properties

- Colour → excitation and emission spectrum
- Molar extinction coefficient
  - ability of the molecule to absorb light
- Quantum yield
  - fluorescence emission efficiency
  - ratio of the number of photons emitted to the number of photons absorbed
  - often sensitive to the environment of the fluorophore
- Quenching and photobleaching
  - reduce the levels of emission
  - molecular structure and environment affect

How to connect the antibody/protein to the fluorophore?

- Numerous commercial secondary antibodies available
  - Alexa dyes available as at least anti-mouse/rabbit/sheep/goat/guinea-pig,...
- Most of the dyes can also be bought separately to label the antibody/protein yourself
  - dye can be pre-“activated”
    - amine-reactive → binds to primary amines
    - thiol-reactive → binds to cysteines
    - etc...

Choosing the fluorescent protein

- The fluorescent protein (FP) should be expressed efficiently and without toxicity in the experimental system
  - Some FPs may not fold efficiently at +37 oC
- FP should be bright enough to provide sufficient signal above autofluorescence
- The FP should have sufficient photostability for the duration of the experiment
- If the FP is used as a fusion protein, then the FP should not oligomerize
- The FP should be insensitive to environmental effects
- In multi-colour imaging, the set of FPs should have minimal crosstalk

Properties of Different Green Fluorescent proteins

<table>
<thead>
<tr>
<th>Protein (Acronym)</th>
<th>Excitation Maximum (nm)</th>
<th>Emission Maximum (nm)</th>
<th>Molar Extinction Coefficient</th>
<th>Quantum Yield</th>
<th>In vivo Structure</th>
<th>Relative Brightness (% of EGFP)</th>
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</table>

Shaner et al. Nature Methods 2005
Choosing the fluorescent protein

Cyan: Cerulean
Green: Emerald or EGFP
Yellow-green: mCitrine, Venus or Ypet
Orange: mOrange or mKO
Red: mCherry
Far red: mPlum

Additional info:

http://www.microscopyu.com/
http://www.olympusmicro.com/index.html