Microscopy Sample Preparation Guidelines

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Purpose: The principles behind sample preparation are transversal to all types of samples, from bacteria to plants. These guidelines will serve as a checklist of practices to follow during experimental design to ensure the most accurate results will be achieved while minimizing errors and reagent waste.

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Bacterial Imaging Preface

As indicated by the name of our unit, Bacterial imaging Cluster (BIC), most of our microscopy experimental design and sample preparation expertise comes from bacterial samples, fortunately the principles behind them are transversal to any kind of sample.

Bacteria in general make good fluorescence microscopy samples due to their high transparency, the readily available array of genetic tools for specific fluorescent protein expression, and the large amount of commercial fluorescent dyes for target labeling. In combination with fast replication cycles that lead to large specimen numbers in a single sample it translates into robust results.

The Proper Coverslip

Using the correct coverslip thickness is one of the cheapest ways of improving your images. Microcope objectives are manufactured with a specific coverslip thickness in mind, usually indicated on the objective barrel. Utilizing the incorrect objective leads to optical aberrations and signal intensity loss, the higher the Numerical Aperture (NA) of the objective the more sensitive it is to deviations from the optimal coverslip thickness.

Coverslips are divided in classes from #0 to #4 by increasing thickness as shown on the table to the right.

Coverslip #	Thickness
0	0.085 - 0.13 mm
1	0.13 - 0.16 mm
1.5	0.16 - 0.19 mm
1.5H	0.17 - 0.18 mm
2	0.19 - 0.23 mm
3	0.25 - 0.35 mm
4	0.43 - 0.64 mm

Currently all our objectives are optimized for **0.17 mm** coverslips, therefore they require #1.5 or #1.5H coverslips. For the widefield systems #1.5 coverslips are sufficient, however for high or super resolution systems, such as the confocal, #1.5H should be used. The H denotes high-precision coverslips, meaning the thickness variation is subjected to a more rigorous quality control.

Sample specifications

There are physical limits as to what can be imaged and how well it can be done using microscopes. Therefore, it is very important to be aware of the sample's dimensions, transparency, and natural autofluorescence as they can heavily influence image quality and resolution.

Dimensions & Transparency

Sample dimension and transparency are closely related as a large dense sample can be divided allowing the imaging of samples that would be too large to be fully observed on a microscope under normal circumstances.

The ideal microscopy samples are mostly transparent, allowing the excitation light to penetrate into the samples and the emitted fluorescence to return to the objective with small interference and signal loss. There is also a physical limit on how far excitation light can penetrate a sample, leading to a clear loss of resolution and signal intensity the deeper into the sample the focal point is situated. The LSM 880 confocal system can achieve an imaging depth of 100 µm, with optimal samples and imaging conditions. The ideal samples for microscopy are single cells, cell monolayers, and small organisms. When imaging bigger more complex samples transparency can be increased using histological sectioning or sample clearing protocols.

Autofluorescence

Autofluorescence is the natural emission of light of biological structures after absorbing light without any added fluorescent markers. Depending on the sample, this can be used instead of a fluorophore to obtain a general overview of cells or presence of organelles.

Example 1 - C. difficile cytoplasm has green autofluorescence that can be used to count cells on systems without transmitted light contrast techniques.

Example 2 – Identify the presence/absence of chloroplasts in plant parts by chlorophyll red autofluorescence.

When using a fluorophore within the same excitation/emission range of your autofluorescence ensure its intensity is strong enough to be easily distinguishable from the autofluorescence intensity by making control samples without the fluorophore.

Fluorophore Selection

The first step is selecting fluorophores compatible with the excitation light sources and emission filters of the microscope. Check the specifications for each BIC microscope at www.itqb.unl.pt/bic.

When selecting multiple fluorophores for the same sample it is important to guarantee a minimal spectral overlap between both. Excitation and emission wavelengths for fluorophores can be found on their respective datasheets, or on online resources such as the FPbase Spectra Viewer.

Using a spectra viewer is an economic way to test new fluorophore combinations on your samples or compatibility between your current staining protocols and available microscopes since it does not require wasting expensive reagents, samples or microscope time. Observing spectral overlap using these tools also helps planing how many control samples should be prepared to exclude crosstalk between fluorophores.

We recommend utilizing the FPbase Spectra Viewer as it is an in-depth tool that has currently collected almost 600 fluorescent protein spectra, while also containing information on protein sequences and structure for most of them. It also has available an extensive list of emission filters and light sources, allowing users to simulate the excitation efficiency of their fluorophores using the available microscope specifications.

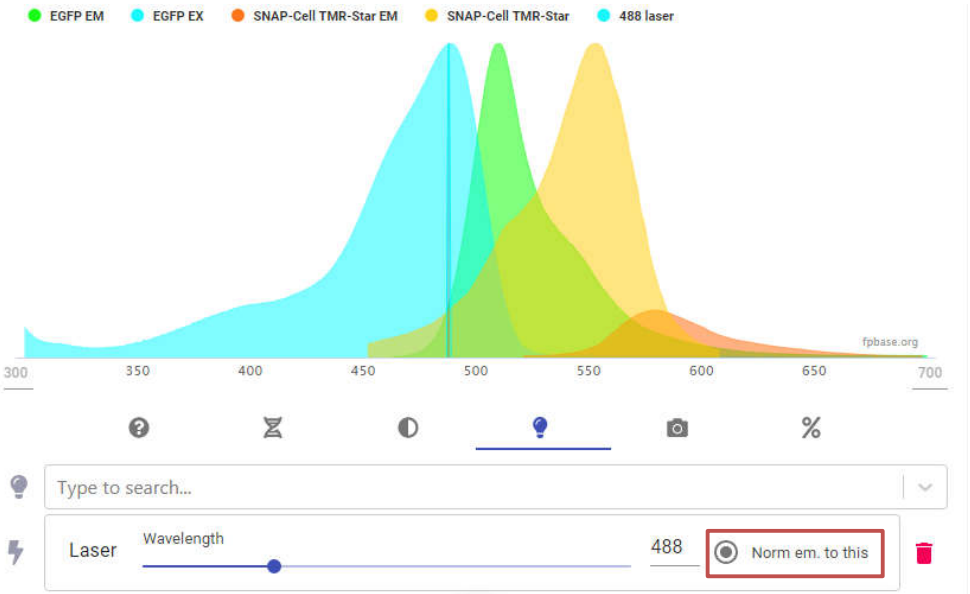
How to use FPbase

The overall FPbase Spectra Viewer user interface is very intuitive, with separate tabs for fluorophores, emission filters, light sources, detectors, and efficiency. Under detectors you can find a list of microscopy cameras and their respective detection quantum efficiency curves. In the efficiency tab you can find the percentage of collection efficiency of the selected filters for the selected fluorophores

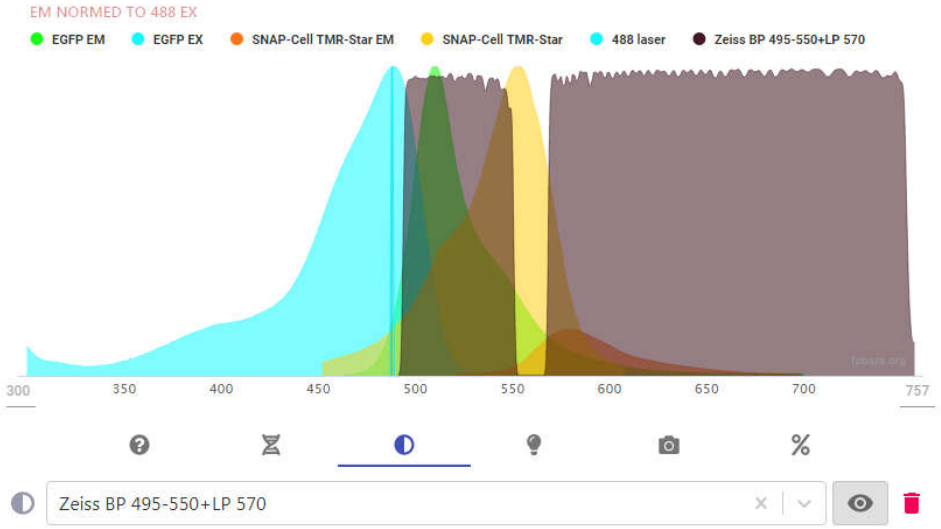
1. Search for your fluorophores on the All or Fluorophores tab, where fluorophores are divided into two categories, fluorescent proteins and dyes

2. The selected fluorophores excitation/absorption and emission curves are displayed in the graph and can be individually toggled, on top of the graph or on the right of the dropdown menus. (EGFP and SNAP-Cell TMR-Star shown)

3. Under Light Sources select Add Laser and insert the desired wavelength, then select normalize emission to this so the emission curves are adjusted to the laser wavelength. Both EGFP and SNAP-Cell TMR-Star are excited by the 488 nm laser, but as expected it is much more efficient on the EGFP as the laser wavelength is on the peak of the protein's excitation curve.



4. Under the filter tab you can search for the confocal Airyscan associated filters to test which is better for each fluorophore while excluding emission from any other fluorophores present. This displays the filter range over the already displayed fluorophore spectra. If the filter is not available on the dropdown list a custom filter can be created to the desired specifications. For example, the Zeiss BP 495-550 + LP 570 filter is a poor choice for the EGFP in this combo of fluorophores as it allows the detection of the SNAP-Cell TMR-Star that can be excited by the 488 laser.



Controls: Is this staining real?

The only way to guarantee without doubt that the observed staining in a sample is real is by performing controls. They can disambiguate weak signals from autofluorescence, and clear suspicions of bleed-through from spectrum-adjacent fluorophores. **Controls should be made at the beginning of experiments** so any issues can be addressed promptly, since no one wants to perform a lengthy and expensive experiment only to find out that controls discredit all the acquired results.

Some bleed-through or autofluorescent signal being present in controls does not mean the experimental protocol must be drastically altered. If this "bad" signal has a different enough intensity to the actual labeling it can be excluded from analysis by determining an intensity threshold from which it is considered actual signal. Of course, such a threshold will then have to be the same across all experimental images, so no bias is introduced.

First image the sample with all the labeling and adjust the settings for its optimal acquisition, then use these settings to image all the controls.

Unlabeled control

This is the easiest control to make, simply do not add any labeling to the sample, mount as usual, and then image it with the same settings as the experiment. If the results are the same as what is seen in the labeled sample, then the labeling is likely not working or too weak to suppress the autofluorescence.

To resolve this quenching reagents can be added to samples, or if the autofluorescence is distinct enough from actual signal just take it into account during analysis, as stated above.

Non-specific binding control

When performing an immunolabeling assay, a control with only the secondary fluorescent antibody should be performed to detect any unspecific binding or aggregation.

To counteract these phenomena, try to adjust the time of the blocking and washing steps.

Single label controls

A single label control is done by labeling the sample with each of the utilized fluorophores individually and imaging each single-labeled sample with the previously set experimental settings. Ideally each fluorophore should only show signal in its own channel, if there is signal in a different channel than the expected then there is signal bleed-through. If the fluorophore selection guidelines above were followed there should be almost no bleed-through detected in these controls, but they should be performed at least once at the start of the experiment.

Just like with autofluorescence, if the bleed-through signal is weak and easily distinguishable from the real signal it can be mostly ignored and just considered during image analysis.

Strong bleed-through can have many causes and the best counter measure is proper fluorophore selection for the available filter sets, making sure there is minimal emission spectra overlap.

Sample Mounting

Cell suspension

Cell suspensions can be mounted on cleaned (70% ethanol) microscope slides by placing 2 µl of the suspension directly on the slide and placing the coverslip on top carefully to reduce bubbles in the sample. This method is a fast way to mount the cell suspension, but the cells will most likely be very mobile in the fluids unless gently squashed by the coverslip into the slide, which could lead to cell morphology artifacts. To reduce cell squashing, you can use microscope slides with a concave surface where the cell suspension can be placed, or create your own small well by cutting a hole in parafilm placed on the slide or two coverslips to create a step on either side of the top coverslip.

Another option is creating a thin agarose pad on the slide where your cells can rest, this method also reduces media evaporation maintaining the cells alive for longer periods.

1. Dissolve 1.7% (w/v) agarose in ddH_2O, or culture media for long term experiments/sensitive cells, using a microwave with care not to boil or burn the solution.
2. Equilibrate the 1.7% agarose solution at 50°C before spreading 800 µl of it on a clean microscope slide. Use a second slide gently placed on top to evenly spread the agarose along the surface.
3. Allow the agarose to polymerise for at least 5 minutes at room temperature before gently separating the slides maintaining the agarose pad on one of them.
4. Trim the edges of the agarose pad so the edges of the slide (~1 cm each side) are free of agarose.
5. Apply 2 µl of the cell suspension and gently place the coverslip on top.

For easier and more consistent agarose pad sizes commercial frames, such as Gene Frame from Thermo Scientific, can be used.

Cell monolayer

Cells grown in a monolayer must be observed where they are grown, this usually means growing them directly on a coverslip and then mount the coverslip on a slide. The main problem with this is growth media residue on the cell free side of the coverslip will interfere with image acquisition, even cleaning the coverslip with 70% ethanol is not completely effective as coverslips are easily damaged.

An alternative for inverted microscopes, such as our Zeiss LSM 880 confocal, is growing the cells directly on coverslip bottom dishes or chambered coverslips, this not only removes the residue on the cell free side of the coverslip it reduces handling of the monolayer. If properly sealed using these chambers can easily allow for prolonged studies using the same cells. These solutions are geared towards cell culture imaging and are commercially available from companies such as Ibidi and Willcowells, that usually can also provide free samples of these products.

Currently our system can only fit 35 mm diameter dishes and 25x75mm chambered coverslips, which are the size of a regular microscope slide. When acquiring these products confirm they are adequate for inverted microscopy and the proper size for our systems.