

Quickstart guide to Good Imaging Practice

What is this about?

When spending long hours in dark rooms, learning a lot about complicated machines and missing one or the other beer session or Champions League match, it is desirable to get the most out of it: As much biological information as possible. Using the correct settings during image acquisition allows later extraction of this information through image comparison or computerised analysis and quantification. This Quickstart Guide to Good Imaging Practice is aimed at helping to get good quality images the microscopist can rely on.

Planning an imaging experiment

One consideration is crucial to maximise the biological information contained in microscopic images: The more standardised sample preparation and image acquisition, the more useful information your final image files will contain. Some important considerations are:

Culture conditions

Whatever organism, reliable results can only be expected if the sample is perfectly healthy to start with. Optimise culture conditions and observe them regularly. This is particularly important for transfections or any other rough treatment. FACS sorting for viable, fluorescent cells can help in this case.

Consistent conditions throughout sample preparation

As obvious as this may sound, this can be a tricky task particularly when upscaling from just a few samples to e.g. 24-well plates, simply due to the time for each pipetting step. Possible sources of variation are:

- different fixation and permeabilisation times
- uneven coating of coverslips
- uneven staining with antibodies

- uneven drying of coverslips before mounting (drop of water at the edge)
- fixation can be particularly variable between experiments (always make paraformaldehyde fresh)

Controls!

Because of so many variables affecting the final images, the proper controls are essential and must be included in every staining or transfection experiment:

- negative controls (no transfection or staining with secondary antibody only): cells can have high autofluorescence under certain conditions, and this is the only way to control it
- single-fluorophore controls for each fluorophore, or for combinations of more than 2 fluorophores a control lacking one fluorophore for each

Fluorophore combinations

Check before starting an experiments which fluorophores can be imaged on which microscopes. A range of labelled secondary antibodies are available for trial from the facility, and fluorescent protein vectors for cloning can also be provided (*see below: Facility Trial Stock*)

Image Acquisition

Choice of equipment

- make sure the specifications of the equipment match the samples (excitation / emission ranges, speed and incubation for live experiments)
- special dishes and plates for live imaging can be borrowed from the facility for testing, as well as some mounting media and dyes (*see below: Facility Trial Stock*)
- don't switch between microscopes within experiments, microscope setups are never identical and so are the images produced – but don't be scared to switch to the best one for each experiment: with the proper training it will be easy

Settings affecting image content and quality

These are general considerations true for every microscope in the facility, both confocal and widefield fluorescence. A good understanding of the basics of image acquisition and processing will help to produce good-quality pictures and reliable biological data.

Probably the most important factor affecting the result of microscopy experiments is the experimentator's personal observation and experience, so:

- always observe samples in transmitted light, this gives a huge amount of information on viability, size, shape, structure, cell density, granularity,.....
- observing fluorescence down the eyepiece provides invaluable information, some never seen through the camera:
 - relative location
 - absolute and relative brightness (settings can differ)
 - direct comparison between fluorescence and transmitted light
 - auto- and background fluorescence (not visible on the screen if set up properly)
- be aware that the expression level of a protein almost always affects its function (after all, that's the whole point of gene regulation):
 - Unphysiologically high overexpression of proteins almost inevitably results in unspecific interference with cellular signalling: Extremely bright cells / samples should thus be avoided (see below: gain / offset settings).
 - Because this is specific for each protein, determining the maximum physiological expression level is down to the microscopists personal observation.

Hardware settings directly affecting image data

The more standardised the conditions for image acquisition, the more biological data the files contain. First, some explanations:

Light intensity

It is obvious that light intensity (laser power, filters) affect an image, so they must be kept constant throughout an experiment.

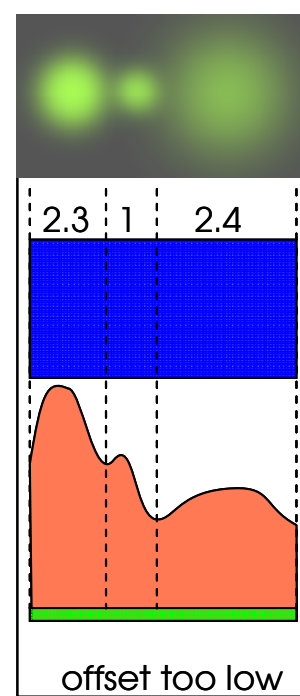
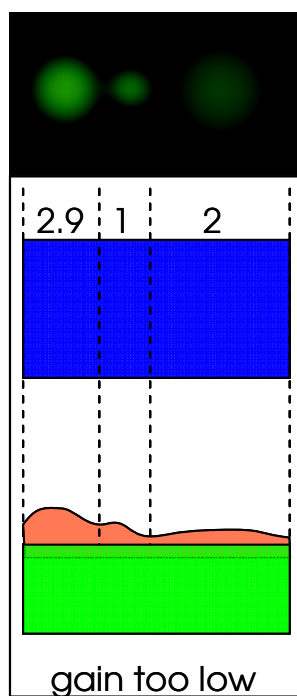
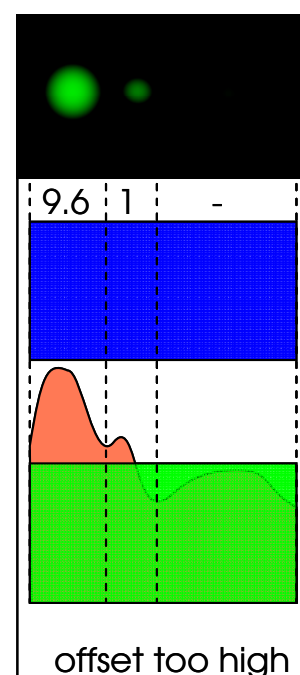
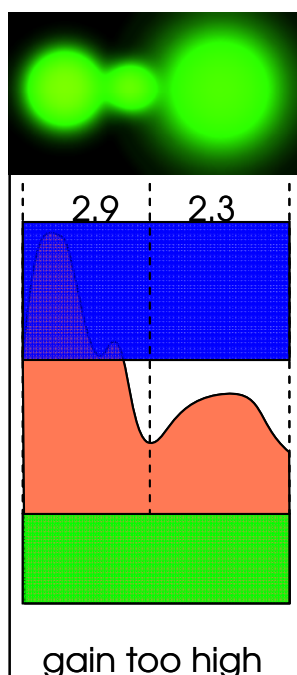
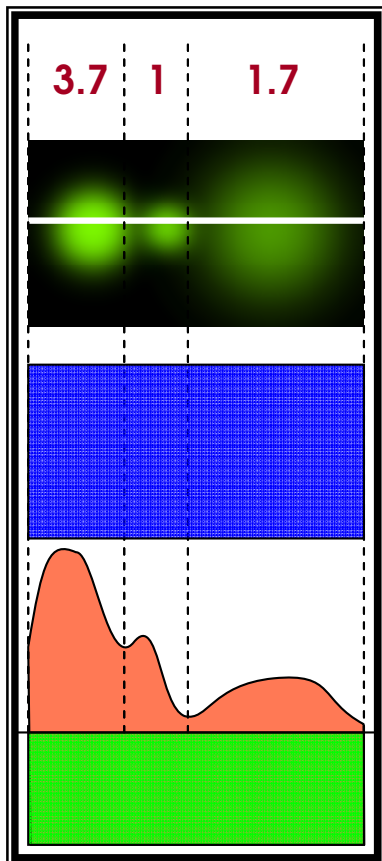
Gain / Exposure time & Offset

Gain (+ exposure time on a camera) and offset dictate the signal intensity and noise level of an image, they are the key settings on a microscope:

- the gain adjusts the signal amplification at the detector / camera sensor:
 - it amplifies the signal and the noise → the lower the gain, the better the signal-to-noise ratio
 - longer exposure times on a cooled camera have less effect on the noise level, so are preferable
- the offset is the minimal signal intensity detected by the sensor, the higher the offset, the more background (and signal) is cut off

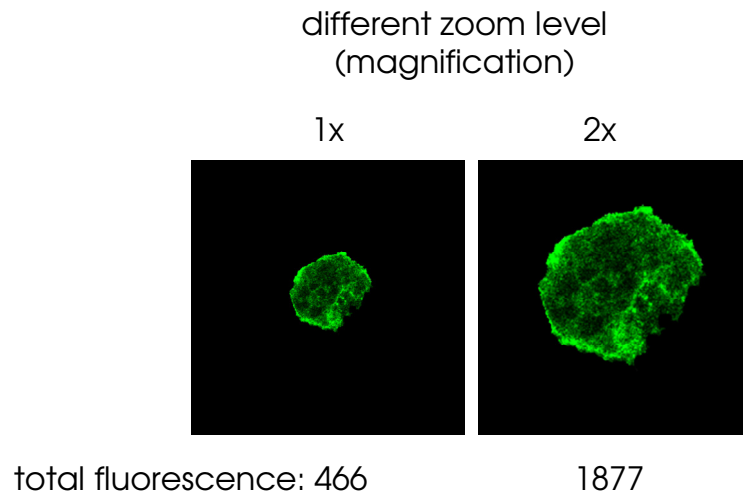
Gain / Offset (and exposure time) must be set in a way that none of the signal is lost, but the whole available range of brightness levels (dynamic range) is used for imaging. The following drawings illustrate the effects of Gain / Offset settings on image quantification:

correct settings



Magnification / Zoom level

It is important to remember that not only the gain / offset settings, but also the magnification and zoom level (on a confocal microscope) affect image quantification. For illustration the quantification of the same cell at two different zoom levels:



Workflow to set up a microscope

Considering these effects of settings on image properties, the following workflow shows how to get to good instrument settings:

- start with a positive control
- looking down the eyepiece, look for a bright sample at the top physiological range of brightness (*check brightfield for viability*)
- set software to show saturation on screen
- start acquisition
- increase GAIN (on widefield: start with ACQUISITION TIME) until saturated pixels appear in the interesting area of the sample, then reduce until they just disappear (*on widefield microscopes, only use GAIN if a longer acquisition time is not sufficient*)
- lower the OFFSET threshold until black pixels appear in the background, then revert until they just disappear
- on confocal: set the zoom to a good value
- save the settings and apply for image acquisition throughout the experiment (do not change them!)
- label / note any samples taken with different settings, they must be excluded from any quantification

Data publication

Metadata

The following metadata (acquisition parameter) should be included in the *Materials & Methods* section of a publication:

- sample preparation (live or fixation protocol, dish, stainings, ...)
- microscope type (widefield, confocal)
- objective
- fluorescence excitation wavelengths (laser line or filter)
- fluorescence emission wavelengths
- scale (best as scale bars) and stack sizes
- live images: acquisition speed

In PhD theses and similar, more detailed information should be given to allow repetition of the experiment on the same equipment (gain, offset, exposure times, pixel size, laser settings). The easiest way is probably to use the metadata information from the original image file as a table in an appendix.

Images

The most important thing is that all images are acquired, adjusted and presented in exactly the same way, including all relevant controls (negative and positive!). Scalebars and pseudocolour calibration bars are useful tools to optimise the information value.

The images should be presented in a way to make the result easily visible to a maximal number of potential readers. Essential issues to consider are:

- How does the image look on a screen, when printed in colour or grayscale; ideally the essential information should be visible under all conditions.
- How is the image seen by a colour-blind person (red/green double stainings can hardly be interpreted)? 10% of men are colourblind do some degree, maybe also the reviewer.
- Is the image visible when scaled to its actual printing size?

The easiest way of getting around most complications is to show images in black on white (grayscale fluorescence, inverted). Unusual but very effective.

Another efficient way is to use pseudocolours to improve visibility of fine differences. It is just essential that all images are shown with identical pseudocolour settings.