Quantitative Fluorescence Symposium
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TIPS AND TRICKS

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- **Finding your sample on confocal:**

Conventional brightfield imaging:

- generally causes little damage to sample
- DIC will improve contrast to low-contrast image, especially good for live cells

Wide-field epifluorescence

- be careful as this will cause your sample to bleach quickly
- be aware that neutral density filters should be used to reduce intensity of uv lamp

- **When using Confocal Mode to locate cells:**

How to reduce damage to cells

- use low power setting for laser, increase gain (voltage) to 'brighten' image
- increase scan speed, reduce image size (eg 256x256 pixels) while move around sample
- be aware that when you use Transmission mode on the confocal that you will still bleach your sample as you are still exposing sample to laser!

Leica SP5 has a 'find focus' button which can be useful (but this won't actually find your sample!!)

- **Standard samples** are useful to establish relative changes in laser intensity and general state of confocal laser scanning system (from day-to-day or week-to-week)

Suggestions:

1. Fluorescent solution
2. Fluorescent plastic
3. Latex beads or Qdots
4. Pollen grains
5. Spores (rust)
6. Autofluorescence of hair
7. One of your own immuno samples? – consider issues of storage, age and loss of intensity
8. GFP intensity beads are available –

BD Living Colors™ EGFP Calibration Beads are a set of EGFP (Enhanced Green Fluorescent Protein) standards.
They microbeads are 7–10 mm diameter.
A reference blank population of microbeads is also provided.
These fluorescent standards allow you to quantitate the fluorescence intensity of an experimental sample.
The fluorescent standards consist of four populations of microbeads, each coated with EGFP at a different density.
These beads have fluorescence intensity as well as excitation and emission spectra matching specimens labelled with EGFP.

Available through BD Biosciences Clontech: www.bdbiosciences.com

- **Sobering thought!!**

What is the efficiency of a 'typical' confocal system?
Optimal situation – the very *best recovery* you can hope for is detection of **22% of total photons emitted** by fluorophore!
Remember that fluorescence occurs in every direction, so that the initial loss of fluorescence in directions other than towards the lens is the greatest loss component. After that, it is dependent on the efficiency of transmission through glass filters. The Leica 'glass-less' system may be more efficient in terms of transmission of light through the system, but is still affected by initial limitation of direction of fluorescence.

(data provided by Prof Simon Watkins – based on transmission of light through components of conventional confocal)
How is this figure arrived at…..

Efficiency of collection of light by 1.4NA lens (30%)
Transmission of 1.4NA lens (90%)
Transmission of dichroic filter (85%)
Transmission of final barrier filter (90%)

- Some thoughts on the effect of aberrations can be significant in relation to creating **3D reconstruction of a series of optical slices**

This is even more serious when attempting to make 3D measurements from an image stack!