Confocal versus Widefield – which microscope to use?

This Light Microscope Facility is fortunate in having a diverse range of equipment for you to use.

In the LMF there are confocal point-scanning microscopes (LSCM), widefield microscopes (WFM), an Optigrid structured illumination microscope, and a fast live-cell capture system. A confocal builds up its image rapidly from a series of points, rather like reading words in line on a page to form sentences and paragraphs. A widefield microscope collects all the fluorescence signal at once over the entire field of view, and deconvolves the image to remove fluorescence blurring. Besides the instruments that we have, there are also Nipkow spinning disc confocals and multiphoton microscopes for looking at fast moving and very thick samples.

Which microscope is best suited for your application?

First, how thick is your sample?
Second, is it living and moving: do you need to record dynamic events?

Here are the short, general, answers to which microscope to use:

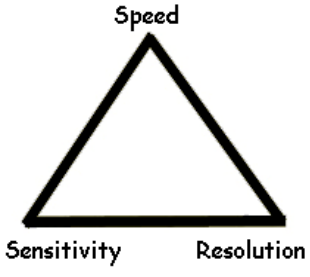
Thick samples (10 – 70 μm) are best imaged using a point-scanning confocal.
Thin samples (1 – 10 μm) are best imaged using a widefield deconvolution microscope.
Photo-sensitive samples should be imaged using the structured illumination microscope.
Very sensitive samples should be imaged using the EM-CCD on the live-cell microscope.
Very thick tissue (70 – 250 μm) should be imaged using a multiphoton microscope.
Moving samples should be imaged with a Nipkow spinning disc confocal or the live-cell microscope.

Points to consider:

High magnification objectives have a limited depth of field - the distance between the upper and lower planes of the in-focus region – which is often a micron or less. Fluorescently-stained specimens are self-luminous. Thus specimens having a thickness greater than three to five microns will produce images in which most of the light is contributed by regions that are not in exact focus. Hence the need to optically section your sample.

1. Do you need live cell imaging – inverted systems are best for this. Can your (fixed) sample be imaged inverted?
2. If the sample is fluid, image with Petri-dishes or well-chambers with a coverslip bottom.
3. Do you need to provide heating and/or CO_2 over any length of time?
4. Do your fluorescent probes match the available lasers and/or filter blocks?
5. Better optical sectioning in LSCM than WFM.
6. If using LSCM, overstain thicker samples to provide better S/N
7. If zooming is important, use the LSCM.
8. Use the widefield microscopes (DeltaVision/Optigrid/live-cell) if your sample is photo-sensitive.
9. WFM with deconvolution is less good for rapidly moving samples.
10. Consider fluorescent protein overlap – LSCM good at spectral separation
11. EM CCD on Leica live-cell fastest instrument but it has limited fluorophore sets
12. Have you got a lot of slides to process – consider the Optigrid.

There is always a trade-off between photo-damage and collecting a high S/N ratio.
Or, to put it another way: between (a) acquisition speed (b) sensitivity and (c) resolution.
A gain in speed involves a sacrifice of both speed and resolution, and so on.



For more a more detailed answer on which microscope to use, here are the advantages and disadvantages of each microscope system compared:

Which microscope to chose? Advantages - Disadvantages of each type of microscopy.

1) Laser Scanning Confocal Microscopy

Advantages

- Non-invasive optical sectioning – smaller PSF than confocal – sharp optical sectioning.
- Reduction of background - Reduced blurring of the image from light scattering.
- Coherent laser illumination reduces chromatic aberration for co-localisation.
- Laser switching – better elimination of cross-talk than WFM.
- Magnification zoom can be adjusted electronically - PMT not limited by a matrix of CCD on WFM.
- Greater control of regions of interest for bleaching – FRAP, FRET.
- Can capture 2-4 fluorophores simultaneously.
- Consider fluorescent protein overlap – LSCM good at spectral separation.

Disadvantages

- Light scattering/refractive index mismatches limits depth penetration to 100-200μm.
- PMT on LSCM noisier than CCD on WFM; less quantum efficiency.
- Laser power noise and fibre optic coupler (change polarization state) leads to artifactual pixel-pixel fluctuations.
- Laser results in very high light intensity – photo-bleaching and photo-toxicity.
- Laser bleaching of sample higher than spinning disc confocal.
- LSCM is slow: confocal image took 10 sec to collect; wide-field (WFM) took 300 msec to collect.
- Real-time collection difficult; need to wait to collect a low-noise signal.
- Samples must be well-stained.

The LSM envelope consists of three main trade-offs: image quality, acquisition speed, and light intensity. Improving one of these variables usually requires sacrificing one of the other variables. For example, higher quality images can be captured if slower acquisition speeds are used. Acquisition speeds can be faster if brighter excitation light intensities are used. Slow acquisition leads to photo-bleaching.

2) Wide-field microscopy (deconvolution and live-cell microscope)

Advantages

- Bright fluorescence signal not attenuated by pinhole.
- High acquisition speed over entire field by CCD with higher quantum efficiency than PMT.
- Broad-band mercury arc lamp excites a more fluorophores over visible spectrum.
- Less photo-bleaching, photo-toxicity, and specimen damage.
- Useful increase in S/N and contrast when speed and sensitivity requirements preclude collection of Z-stack.

Disadvantages

- High non-specific background labeling is common.
- Out-of-focus blurring degrades image and reduces visibility of fine detail.
- More time-consuming for deconvolved image output.
- Deconvolution to sharpen images requires accurate knowledge of PSF, measured or theoretical.
- Sensitive to spherical aberrations: changes in temperature & coverslip thickness.
- No zooming function.
- Region of interest function, if present at all, is much less sophisticated than LSCM.
- Deconvolution is often not so successful with live specimens. Any movements that occur in the specimen, while a Z-stack of optical sections is being recorded, will abrogate successful deconvolution.

Works best with 1-15 μm samples. The DeltaVision system gives very good resolution with thin samples, It also has an extremely accurate and stable stage, and the multiple point visiting function. WFM images are much less noisy for three reasons. (a) First, illumination is delivered in parallel to all regions of the sample simultaneously, eliminating pixel-to-pixel differences in delivered illumination. (b) Second, the CCD has ≈ 4-fold higher detection efficiency than the photomultiplier tubes of the laser-scanning confocal microscope, increasing the number of detected photons by 4-fold. (c) Third, the CCD read-out electronics add less noise to the signal than the LSCM electronic circuitry.

3) Structured Illumination – Optigrid/Apotome

Advantages

- Simpler to use than a LSCM, and faster to use overall when repeatedly imaging through a set of slides.
- Faster acquisition than a LSCM - will do about 9 frames per second at full resolution, and up to video rate (30 fps) with binning and cropping.
- Better than the LSCM for imaging photo-sensitive samples (photobleaching leads to a low signal-to-noise ratio).
- With the Xenon metal-halide light source, the Optigrid can image many more fluorophores, and is limited only by the filter blocks, and not the availability of the lasers.
- Cheaper to use than LSCM or Nipkow Spinning disc (no laser sources).
- If collecting a single fluorophore – better than LSCM (laser switching to eliminate cross-talk does not apply).

Disadvantages

- Optigrid cannot do region of interest (ROI) photobleaching and photo-activation.
- Cannot change the thickness of the optical section. Depth of field fixed by objective used.
- Needs care in setting the Illuminated Field Diaphragm for a good S/N ratio.
- The confocal is good for excluding out-of-focus fluorescence from strongly-scattering samples (like fat tissue in zebrafish embryos), the Optigrid-Apotome is not good for this. Neither is the Optigrid-Apotome good for thick largely-homogeneous structures (e.g. bone). Also the Optigrid-Apotome is not so good for samples which exhibit very strong and regular structures.

4) Nipkow Spinning Disc confocal microscopy

Advantages

- Much higher frame rate than LSCM - Good for long-term time lapses.
- Image collection is routinely at 10-15 frames per second, and can exceed video rate for small frames, or if the fluorophores are bright enough and the CCD camera sensitive enough.
- High acquisition speed over entire field by CCD with high quantum efficiency.
- Less photobleaching, photo-toxicity, and specimen damage.

Disadvantages

- Some cross-talk between adjacent pinholes/microlenses.
- Non-modular: Can only be matched to one objective magnification, normally 100x.
- Less optical sectioning versatility than LSCM.
- No zooming function.
- Region of interest function, if present at all, is much less sophisticated than LSCM.
- Can only bin to increase gain, and thus loose resolution.
- The center of the field is always brighter than the edges.

The Nipkow disc microscope is particularly suited to high speed and light-sensitive live-cell imaging. It is not capable of instrument zooming, so it is not possible to exploit the full resolving power of the objective, and the optical resolution cannot be matched to the Nyquist sampling requirements of the object. A spinning disk system with optimally-sized pinholes will behave almost as a point-scanner if the sample is fairly thin (say, less than 10 μm), since the cones of light above and below the focal plane will not overlap. Cross-talk will increase as the thickness of the sample increases, say above 50 μm.

5) Multiphoton microscope

Advantages

- Low photobleaching outside focal volume. Focal volume much less than LSCM.
- Increased depth penetration - Multiphoton best for thick tissue imaging.
- Longer wavelength illumination is scattered less – collects much more emitted signal.

Disadvantages

- Very high costs – intensive service and running costs.
- Thermal damage to chromophores which absorb in the IR.