Concise Overview of Common Light Microscopy Modalities

Many different types of light microscopes are available, each with their own strengths and limitations. A main tenant of good microscopy is to select the right tool (microscope) for the job at hand. Please contact us (microscopy@umich.edu) to help you pick the best microscope for your project.

Wide-field Microscopy:

Transmitted Light: Transmitted light techniques use a lens termed the condenser to shine (white) light through the sample. Contrast in the image is then generated via either light absorption (e.g. bright-field), light scatter (e.g. dark-field) or by refractive index changes in the sample (phase, DIC). A lens on the opposite side of the sample, termed the objective, collects the transmitted light and forms an image. Transmitted light techniques are sensitive to bulk optical or chemical properties of the sample but rarely allow observation of specific molecules.

Advantages:	Simple light path.
Disadvantages:	Little / no molecular specificity.
Common Applications:	Low-medium magnification imaging of fixed cell culture monolayers or thin (<5 um) tissue sections.

Epifluorescence: Epifluorescence illuminates (excites) a fluorescent sample through the objective and then the same objective used to collect fluorescent emissions traveling in the opposite direction. An overarching advantage of fluorescence is that sensitivity can be very high (down to single molecules) and many biomolecules can be specifically tagged with fluorescent dyes, even in living cells. Wavelength selective filters provide a small range of excitation wavelengths, while other filters sort out the longer wavelength fluorescence emissions before they reach the camera. A major drawback of epifluorescence is that out-of-focus emissions are also collected, causing 'flare' that greatly degrades image contrast (and therefore resolution).

Advantages:	Fast, gentle, sensitive, relatively simple light path.
Disadvantages:	Out-of-focus emissions degrade contrast, no optical sectioning (requires physical sectioning with a knife).
Common Applications:	Low-medium magnification imaging of cell culture monolayers and thin (<5 um) tissue sections.

Confocal Microscopy:

A main drawback of epifluorescence microscopy is that out-of-focus light greatly degrades image contrast. The ability to remove out-of-focus light is a tremendous imaging advantage, and many technologies have been developed to create optical sections.

Point-scanning confocal: Illuminates a single, diffraction-limited spot (~200 nm diameter) in the focal plane, while oscillating mirrors traverse the point back and forth in a raster pattern across the sample to create an image sequentially over time. Light emitted (or reflected) from each point then travels back through the objective, the mirrors, and an adjustable pinhole before reaching the detector. Optical sectioning occurs because A) the illuminating spot of light spreads into a cone above and below the focal plane (so the flux is reduced) and B) the remaining out-of-focus emissions generated are blocked by a 'pinhole' located in a conjugate image plane (i.e. the sample and the pinhole are 'confocal').

Advantages:	Optical sectioning provides crisp images and allows direct acquisition of 3D images.
Disadvantages:	Relatively slow (< 1 fps), relative insensitive (10x less sensitive that epifluorescence), relatively high laser powers.
Common Applications:	High magnification imaging of fine subcellular details, z-stacks for 3D reconstructions, imaging into thick (5-100 um) tissue sections.

Spinning disk confocal: Uses the same principle as point scanning confocal to block out-of-focus light, but the process is highly parallelized, based on an a specially arranged array of pin holes on a disk. As the disk spins, the image of the pin holes sweeps across the field to build up an image over time.

Advantages:	Optical sectioning at high speed (>1 fps) and with more gentle laser powers.
Disadvantages:	Pinholes not adjustable and are closely spaced so optical sectioning is not as good as with point scanning confocal.
Common Applications:	High magnification imaging live cell imaging of fine subcellular details in cell culture monolayers.

Multiphoton Microscopy:

Multiphoton: Like confocal, point-scanning multiphoton microscopy also creates an optical section, but it does so using entirely different principles. Multiphoton microscopy relies on very short (100 fsec), intense, bursts of light to induce an effect called multiphoton absorption, where multiple (usually two) photons interact simultaneously with an electron in a dye to trigger fluorescence excitation. If two photons provide energy, each photon needs only half the energy for excitation (compared to single photon excitation). Thus, the excitation wavelength can be about twice as long, usually in the near infrared (700-1000 nm) where tissue is more transparent. Additionally, multiphoton absorption is extremely improbable except where the light is most focused (concentrated). As a result, no out-of-focus fluorescence emissions are ever generated and all emissions—even those that have been scattered—can be used for imaging (no pinhole is needed). Together, these effects contribute to multiphoton's ability to image far deeper (5x) into scattering tissues than is possible using confocal microscopy.

Advantages:	Deep (100-500 um) imaging into highly scattering samples.
Disadvantages:	Relatively slow (<1 fps), practically limited to blue and green dyes, may cause unanticipated autofluorescence.
Common Applications:	Intravital imaging of adult tissues or large organoid cultures.

Super Resolution Microscopy:

Traditionally, resolution (the ability to distinguish two small objects) in light microscopy has been limited by wave interference patterns resulting from diffraction. Although diffraction is unavoidable, various strategies have been invented to circumvent the limitation that diffraction places on resolution. Together, these are termed 'super resolution microscopy' although each strategy works very differently and has different strengths and limitations. The main types of super resolution are localization microscopy (PALM/STORM/SRRF), structured illumination microscopy (SIM), and stimulated emission depletion microscopy (STED). Expansion microscopy protocols have largely supplanted the value of super resolution for fixed-cell imaging, though PALM and STORM are still very powerful when used along with particle averaging.

Structured illumination microscopy: SIM excites the sample with various sinusoidal patterns of light and then uses complex math to solve for image details that are beyond the diffraction limit by up to a factor of 2 (i.e. ~100 nm resolution is possible). The math involved is beyond the scope of this brief description, but is analogous to the modulation and demodulation processes used in early radio broadcasting to transmit audio through an antenna. SIM can be performed in TIRF-mode, 2D-mode, or 3D-mode. TIRF-SIM is relatively fast (<4 fps) and gentle, but is limit to the TIRF zone (<200 nm from the coverslip). 3D-SIM has improved resolution in xy and z, but is slow (>10 sec for smallest possible volume) and phototoxic. It also usually only works well within 5 um of the coverslip surface.

Advantages:	Roughly 2x improved resolution, no special dyes required (but brighter is better), TIRF-SIM is ideal for live cell imaging.
Disadvantages:	Out-of-focus light is a major problem, so sample must either be thin (<5 um) or sparsely stained. Sample must be stationary during acquisition of each raw data set. For 3D-SIM, refractive index matching of sample to immersion is very important.
Common applications:	Getting highly detailed images from the bottom portion of cell culture monolayers.

Stimulated emission depletion microscopy: STED is a point-scanning technique that the shines an annulus (doughnut shape) of intense light to cause emission depletion ring within the sample. A (Gaussian) focused spot of excitation light the excites the remaining dye that was not emission depleted. Most of the emissions come from the center of the annulus, which is smaller than the diffraction limited spot diameter.

Advantages:	Can exceed the resolution of SIM in some cases, can scan small regions very rapidly (msec).

Disadvantages:	Special dyes are required to match the depletion laser and withstand the high laser powers involved. Generally too phototoxic for live imaging.
Common applications:	High resolution imaging within 10 um of the coverslip.

Localization microscopy: PALM/STORM rely on collecting a large sequence of sparse, single-molecule emissions, usually in TIRF mode. An algorithm is then used to estimate the location of each dye molecule from its image. The sum of all of these localizations creates a high resolution image of the sample. Special dyes and buffers are also required to achieve sparse molecular imaging. When the sample consists of many instances of the same structure (e.g. viral particles), particle averaging can be used to create a model of the 'average' particle at resolutions <10 nm, by far the highest of any optical method.

Advantages:	Can exceed the resolution of SIM in most cases, can scan small regions very rapidly (msec).
Disadvantages:	Special dyes and buffers are required. High laser powers are needed to cause photoswitching. Not live cell compatible.
Common applications:	Very high resolution imaging within 1 um of the coverslip. Molecular counting and structural biology applications.

Written by Dr. Aaron Taylor, BRCF Microscopy Core, December, 2019.