Microscopy and Filters – Technical Tips and Tricks:

Technical tips & tricks which should be considered before performing bright field or fluorescence microscopy:

Basics of Microscopy:

- Resolution is defined as the ability to perceive two points separately. The human eye has a resolution of ~100 µm. Since, in pathology the objects of interest, i.e., the morphology of cells, often are below 100 µm range, optical tools such as microscopes are necessary to enlarge the resolution of the human eye. A microscope is comprised of concave lens sets that are sequentially set up as several magnifying glasses after another. The final magnification is the product of each single magnification of lenses in row.

- The resolution of a microscope depends on the quality of its lenses. Therefore, high quality glass lenses are used in order to focus rather than scatter light. The capacity to enlarge the resolution is called numerical aperture. A high numerical aperture of at least 1.3 is recommended.

- PLAN objectives are recommended for extended field of view leveling. A plan apochromatic objective is of additional value to avoid color manipulation by the lenses especially in bright field microscopy.

- When using objectives with 63x or higher magnification, it becomes necessary to reduce light beam deflection caused by the transition between glass and air. This is achieved by the usage of, e.g., lens immersion oil (according to the objective) which has a higher density than air and thereby reduces light deflection.

- Coverslips should be ≤0.17 mm thick and made of glass to avoid light scattering and deflection.

Fluorescence Microscopy and Filters:

- For fluorescence *in situ* hybridization (FISH) a fluorescence microscope is required. The set up is composed of a light source emitting high intensity light with a broad spectrum. This light is filtered by an excitation filter that transmits light of a certain wavelength range only and is deflected by a dichroic mirror (beam splitter). The deflected light is focused to the specimen by the objective lens. The fluorochromes are excited and emit light of a longer wavelength than that of the excitation light. The emitted fluorescence light of the specimen goes back through the objective and now passes the dichroic mirror due to the longer wavelength. The light is filtered by the emission filter which defines the wavelength of the light reaching the ocular and which is perceived.

- Fluorochromes emit light of certain wavelengths when excited by light of the respective shorter resonance wavelength (see below). This excitation light is produced by certain light sources as the recommended 120 watt mercury excitation lamp. The light source has a lifetime of approximately 2000 hours and should be exchanged regularly. Otherwise, the signal intensity can decrease.

- Please note that for fluorescence microscopy an immersion oil specially rated for fluorescence microscopy is required because it may harbor autofluorescence. Accordingly, glass slides and cover slips should be suitable for fluorescence microscopy.

- Of particular importance is the choice of filter sets. In general, we recommend hard coated band pass filter sets that are comprised of compatible excitation and emission filters and a dichroic mirror in a cubicle set up. These filter sets have a very long lifetime, are cheaper than the single wavelength specific interference filters and allow broad range wavelength's transmission.

- The chosen filter sets have to be compatible with the excitation and emission maxima of the applied fluorochrome. The following fluorochromes are used in ZytoVision FISH probes:

Fluorchrome	Excitation	Emission	Equivalent to	Available Band Pass Filter Sets
ZyBlue ●	418 nm	467 nm	DEAC	Single ● Triple* ● ● ●
ZyGreen ●	503 nm	528 nm	FITC	Single ● Dual ● ● Triple* ● ● ●
ZyGold ●	532 nm	553 nm	Rhodamine 6G	Single ●
ZyOrange ●	547 nm	572 nm	Rhodamine	Single ● Dual ● ● Triple* ● ● ●
ZyRed ●	580 nm	599 nm	TexasRed®	Single ●
DAPI ●	358 nm	461 nm	DAPI	Single ● Triple* ● ● ●

*Triple band pass filters tend to reduce the light intensity.

Brightfield Miroscopy:

- For chromogenic *in situ* hybridization (CISH) a standard light microscope is required. The set up is composed of a light source, field diaphragm, condensor diaphragm, condensor, object table, objective lenses, ocular lenses and microscopical object. The light goes through the condensor lens and is focused to the object which is illuminated. The passing light is focused and magnified by the following lenses and is perceived. Between the condensor lens and the object a phase contrast ring can be placed. However, this should not be done for CISH microscopy.

- To ensure a good illumination of the specimen, the light beam coming from below the specimen has to be focused to the specimen. This is achieved by manipulation of the condensor lens height. The process to optimize specimen illumination by focusing the light of the light source by a condensor lens is called Köhler illumination.

- Do not use phase contrast rings in CISH imaging. A higher contrast will darken sub-cellular structures that might look like green or red signals. The contrast should be kept as low as possible.

- For CISH Break Apart Probes an objective of at least 63x magnification is recommended.