Guidelines for Image Acquisition for Accurate Colocalization Measurements

References:

Bolte S, Cordelières, FP. A guided tour into subcellular colocalization analysis in light microscopy. ***J Microsc*** *2006; 224:213-232.*

Costes SV, Daelemans D, Cho EH, Dobbin Z, Pavlakis G, Lockett S. Automatic and quantitative measurement of protein-protein colocalization in live cells. ***Biophys J*** *2004; 86:3993-4003.*

1. **Make sure the optical system** in the microscope is well aligned.

2. **Use objectives with high numerical aperture**, preferably immersion objectives to reduce aberrations due to refractive index changes.

3. **Choose fluorochromes** with spectra of unambiguous distinction (excitation and emission spectra of the two fluorochromes well apart to avoid cross-talk and bleedthrough).

   Cross-talk: several fluorochromes are excited with the same wavelength because excitation spectra partially overlap

   Bleedthrough: passage of fluorescence emission in an inappropriate detection channel caused by overlap of emission spectra

4. **Always have single labeled controls** for each fluorochrome.

5. **Avoid saturation of images**, as saturated pixels may not be quantified properly (use color look-up tables—LUTs—to adjust dynamics of grey values).

6. **Perform sequential acquisitions** exciting one fluorochrome at a time and switching between the detectors concomitantly.

7. **Acquire Z stacks** according to the Nyquist criterion (image acquisition in 3-D with appropriate pixel size and Z-step).

   On Zeiss LSM 510 META, select **Scan Control>Z Stack>Optical Slice>Optimal Interval.** If optical sections are different for each wavelength, go to **Mode/Channels** and adjust the **Pinhole** so that each channel has the same optical section around **1 Airy Unit**, but one channel will have to be slightly larger.

8. **Perform deconvolution** to reassign out-of-focus blurred light to its origin.

9. Use **Imaris Coloc** software to measure colocalization (uses the Costes method).