# Data acquisition on the SP5 microscope for deconvolution

Successful deconvolution already starts at the level of data acquisition. It is generally not possible to correct major errors in image acquisition at the level of the actual deconvolution process. This protocol therefore gives a few hints about successful data acquisition on the Leica SP5 microscope prior to deconvolution. It is highly recommended to check also the extensive information on the SVI website.

**How small should I make my pixels?**

The most crucial point in deconvolution is to sample the images correctly, i.e. you need to acquire images with a sufficient number of pixels in order to fully capture the complete optical information available under the concrete imaging conditions. The correct sampling is also termed sampling according to Nyquist (Nyquist sampling, Nyquist rate) and it essentially means that we need to choose the pixel size such that we fully capture the available optical resolution. The pixelsize should be roughly 2.3 times lower than the optical resolution, but should always be calculated using the online tool on the Huygens website. **Nyquist sampling (pixelsizes in nm)** for our three immersion objectives for a 3D stack (x,y,z-Pixelsizes) on the SP5 microscope and **blue (405 nm excitation, 450 nm emission)** or **green dyes (488 nm excitation, 520 nm emission)**.

**63x Water Immersion, 1.2 NA**

| | | |
|---|---|---|
| X: | **42** | **50** |
| Y: | **42** | **50** |
| Z: | **133** | **161** |

**63x Glyercol Immersion, 1.3 NA**

| | | |
|---|---|---|
| X: | **38** | **46** |
| Y: | **38** | **46** |
| Z: | **129** | **156** |

**63x Oil Immersion, 1.4 NA**

| | | |
|---|---|---|
| X: | **36** | **43** |
| Y: | **36** | **43** |
| Z: | **108** | **130** |

**How can I change the pixelsize?**

XY: You can either increase the zoom factor until you have the correct pixel size or take images with more pixels: 1024 x 1024 instead of 512 x 512 or even more; or a combination of both.

Z: The z pixel size corresponds to the interval of your z-slices, which you can choose during the setup of the z-stack.

**Which objective/which immersion medium should I choose?**

1) The higher the NA of the objective, the better the resolution!
2) BUT the **refractive indices** of the **immersion** medium (on objective) and **embedding** medium (on sample) **should be the same** for optimum results.
   Example: Water objective for live cells in culture medium (consists mainly of water, too); exact match of the refractive index. Glycerol objective for glycerol based mounting medium (Glycerol, Mowiol).

3) Mismatches of refractive index are less relevant if you're dealing with rather thin samples; in this case the oil objective can also yield very good results even if there is a mismatch in the refractive indices.
4) Some refractive indices

$\eta_{oil}$=1.515

$\eta_{glycerol\_100\%}$=1.474

$\eta_{water}$=1.33

$\eta_{air}$=1

**How to reduce image noise?**

Image noise should be low. This can be achieved by using bright and photostable chromophores (no FITC, TRITC, use eg. Alexa dyes instead) and rather high laser power. PMT **gain** should be **kept low** since high gain causes noise. Another way to get rid of noise is line and frame **averaging.** The lower the noise the better!

**Where can I find more extensive information?**

*SVI Website:* *http://www.svi.nl/*
*Nyquist Calculator:* *http://support.svi.nl/wiki/NyquistCalculator*
*In order to use the ' Nyquist Calculator' you need to know the **back-projected pinhole size** which can be calculated here:* *http://support.svi.nl/wiki/BackprojectedPinholeCalculator*
*Manual (Password required):* *https://support.svi.nl/wiki/index.php?HuygensUserGuides*
*Huygens software at the MUI:* *http://www.i-med.ac.at/itservices/systeme/huygens/*