Co Localization and Working flow with the lsm700

Samples
-1 slide = mousse intestine, Dapi / Ki 67 with Cy3/ BrDU with alexa 488 .
-1 slide = mousse intestine, Dapi / Ki 67 with Cy3/ no BrDU (but with alexa 488) = control neg alexa 488
-1 slide = mousse intestin Dapi / no Ki 67 (but with Cy3)/ BrDU with alexa 488 = contol neg Cy3
-1 slide = incubate with the 2 secondary antibody alexa 488/ Cy3 and Dapi = control neg neg

The LSM700 inverted or upright microscope, the Pc and the ZEN software, should be open at least 30 to 60 minutes before acquiring a 3d co localization image

Choose the objective

For the SV course, we will acquire the Z-Stack with 2 different objectives, the Plan Apo 20x / 0.8 NA and the Plan Apo 63x/1.4 oil immersion

1) Select the Plan Apo 20x/0.8 Na objectif.

2) Introduce your sample (put 1 drop of immersion oil on your sample if you used the 63x 1.4 Na oil objective).

3) Select the **10 AF 488** filter on the **Reflector** list and open the **RL Illumination** shutter trough the TFT display.

4) Trough the **Eyepiece** finds the region of interest and the focus with the well.

= Now you can check your sample under the scope.

5) When you seen your sample close **RL Illumination** shutter and, tip show **Manuel tools.**

= Build your sequential configuration for Dapi, Alexa488 and Cy3

6) Select **Imaging Setup** tools (here you select the first channel / track) = **Track 1.**

7) Go to the **Light Path** tools and tip **Show all.**
Select the first laser you want to use depend of the staining you want to see… for the course use and selected **405** line laser for DAPI. Increase the laser 405 nm laser @ 2%.

8) Tip **Pmt1** (the PMT1 still active) select the arrow is located under the **Dye** column, and select or search **DAPI** under the **search dialogue, and** select the arrow is located under the **Lut** column, and select the blue color.
Go and select the arrow is under the **Pmt1** dialogue and selected the **SP490** filter.

9) Select the **555** line laser for Cy3. Increase the laser 555 nm laser @ 2%.

10) Tip **Pmt2** (the PMT2 still active) select the arrow is located under the **Dye** column, and select or search **Cy3** under the **search dialogue** and select the arrow is located under the **Lut** column, and select the red color.
Go and select the arrow is under the **Pmt2** dialogue and selected the **LP560** filter.

11) Adjust the **Split** dichroic filter @ 500nm

12) Go to the **Light Path** tools and select **Imaging Setup** tools (here you can add a new track channel select the first channel / track) = **Track +.**

13) Select the **488** line laser for Alexa Fluar 488. Increase the laser 488 nm laser @ 2%.

14) Tip **Pmt2** (the Pmt2 still active) deselect the Pmt1, select the arrow is located under the **Dye** column, and select or search **Alexa Fluor 488** under the **search dialogue** and select the arrow is located under the **Lut** column, and select the green color.

15) Go and select the arrow is under the PMT2 dialogue and selected the **LP490** filter.

16) Select **Imaging Setup** tools and select the 2 Track.

17) Select **Auto exposure.**

=Now the microscope acquire an image containing the Dapi and Cy3 in the first Track (Track1) and Alexa 488 in the second Track (Track 2).

18) Select the **Channel**s blue tool and tip **Show all.**

19) Under **Channels** select **1AU** for a correct pinhole aperture (45.2 Microns). Do the same for the second track.

20) Select the **Acquisition Mode** blue tool and tip **Show all.**

21) Under **Acquisition Mode** select:

a) The **Frame size**, this function determines the number of pixels for which you will use to make an image, this tool will therefore determined the physical size of the image and also the resolution of the pixel, you can start with 1024x1024
b) The **Scanner speed** = 9
c) The **Bit Depth** = 16
d) The averaging **Number** = 8
e) The averaging **Mode** = Line.
f)The averaging **Method** = Mean
g) Increase the **zoom** until the **Pixel Size** correspond to the Nyquist criterion (134nm@ 488nm with the 20x/0.8 and 76nm @ 488nm with 63x1.4 objective)

(Check the resolution rule, page 4)

22) On the Zen software Select **Auto Exposure** button.

= Now you have an image.

23) Under the **Channel** tool deselect **Track2.**

24) You can press **Live** and check if the samples are in the focus and where the signal is the highest in Z.

= Now you can adjust correctly your PMT gain and offset.

25) Select **Continuous.**

26) Tip on the Image area the **Channel color lockup** table icon = the image appears in gray level plus some red and blue pixel.

27) During the scan adjust the **Gain Master** value until no more than 1 pixel appears in red color.

28) During the scan adjust the **Digital Offset** value until few pixel appears in blue color.

29) Do the same procedure (19-20) only with the **track2** = deselect **Track1** and select **Track2.**

= Check the Gain Master value, it would be great if the values is no more than 800V
If you can't produce any red pixel even you increases the Gain Master at 800V, push the Laser transmission value until you produce 1 red pixel.
Be careful don't push to much your laser power (high than 20-30%) because you can burn your stain.

The Bleaching is a very critical issue! Bleaching will bias the analysis procedure. Thus If you push the laser over 20% you should to do a time-lapse experiment with 20-30 images and measure the amount of photobleaching. If more than 5% bleaching is observed the laser intensity has to be decreased.

3d acquisition

30) Tip the **Z-Stack** function = a **Z-stack** blue tool dialogue appears.

31) Deselect one off the 2 **Track** troughs the **Channels** blue tool.

32) Scan in **Live.**

33) Go down with the focus wheel = under the specimen.

34) Select **Set First.**

35) Go up with the focus wheel = above the specimen.

36) Select **Set End.**

37) Introduce the z-step size into the **Interval** value correspond to the Nyquist criterion (571 @ 488nm with te 20x0.8 objective, and 283nm for 488nm with 63x1.4 objective) and do **Enter.**

38) Reselect the 2 **track.**

39) Clik the **Start Experiment** Icon.

= Now the system will acquire a stack of 3D images in sequential mode with optimized parameters.
But remember that you should acquire a z-stack from the 3 negatives controls samples with EXACTELY the same setting that you use for the positive sample

RESOLUTION RULE

The objective resolution is linked to the numerical aperture of the objective, the excitation and emission wavelengths and the mounting media refractive index.
Depending on the scale at which you suppose to observe co localization, you should choose the objective that allows you to reach the good optical resolution. Then, you have to set the voxel size coherently with the size of the objects you are interested in, and with the optical resolution of your objective.

To match the optical resolution of your system you should choose a voxel size which is half of the resolution. The Nyquist theorem states that the smallest resolvable structure (defined by the optical resolution limit) must be sampled (at least) twice in order to record all necessary information. The maximum optical resolution resel* can be defined as the radius of the first dark fringe in the diffraction pattern, or half of the diameter of the Airy disc.

Resel, confocal xy-plane $= \frac{0.44 \cdot \lambda}{NA}$

Resel, confocal axial $= \frac{1.5 \cdot n \cdot \lambda}{NA^2}$

Example l **=** 500nm, n =1.518, NA = 1.4
XY- plane resel = 157 nm Axial resolution = 580nm
With Nyquist criterion the ideal voxel is 78.5 nm in XY and 290nm in Z

*Robert H.Webb, Confocal optical microscopy, Rep.Prog.Phys. 59 (1996) 427-471)

It has to be said that if you are interested in objects significantly larger than the resolution of your objective, you could consider to undersample such to achieve an improvement of the SNR of your image (or to use a lower NA objective as well…). This can be useful especially if you have weak staining.
On the other side, if you are interested in objects with a size at the resolution limit it is important that your voxel size is small enough not to lose resolution. Moreover in this case deconvolution before co localization analysis is a good practice which allows you to gain in image resolution and thus precision for small objects localization.
To deconvolve your image you have to be more restrictive in the choice of the voxel size (refer to this page for the computation of the ideal voxel size if you want to do deconvolution: http://support.svi.nl/wiki/NyquistCalculator).

Co localization and confocal acquisition

Co localisation analysis involves comparison of the spatial localisation of two molecule. These two proteins have either been labeled intrinsically fluorescent by molecular-engineering or indirectly fluorescent using fluorescence immune-cytochemistry.
We are ultimately interested in whether these two proteins occupy the same volume of interest. For some experiments, the volume of interest may be the same cell – are the two proteins expressed in the same cell?
If yes, this is the case then there is no better replacement for manual counting of “double-positive cells”.
The result of co localization will always depend on the volume of interest which is defined via the imaging conditions! The “right” size of the volume is always dependent on the biological question. Strictly speaking co localization is an imaging “artifact”. But nevertheless is it providing valuable information. But on the molecular level there is no equivalence. In other words all coefficients measured are “arbitrary” numbers. Comparison between two samples is only possible if the exact same imaging parameters are used.

In other cases we may want to know if the two proteins occur in the same protein-complex or sub cellular compartment. In this case we are limited by the resolution of the microscope and, in a best case, can only say whether the two proteins occupy the same 200×200×400 nm volume – or voxel. If we want higher resolution, we would have to use the technique of FRET which can resolve interactions of proteins 10 nm apart.

Here we descript an acquisition method where the volume of interest is close to 200nm (XY) x 400nm (Z), this acquisition method also proposed some parameters to avoid a series of traps, that might give you some false results in the end

Objectives

Different type of objective is available. It is absolutely important to choose the good on for your experiment.
You have to be careful if your objective is corrected for **chromatic aberrations** with all the wavelengths you want to use (to avoid a Z shift between colors).
For example an **Apo chromatic** objective is corrected for blue/red/green.

The **oil high numerical (1.4 or 1.45)** objectives have to be use with 170 micron cover slip and are usually designate for cell culture. They have a low working distance (around 80 micron). With this kind of objective you will be able to acquire images of collocation with finest optically resolution you can produced with a confocal microscope.

But if the requirement of the resolution is not so important, you can also use a 20x 0,8 Na objective. Especially for weak signals this might be favorable (less noise).

The ***water immersion Deeping lenses*** are used for sample in Petri dish.
They don’t have a really high numerical aperture, but the fact that are directly in the medium without a cover slip avoid the refraction index mismatch between the sample and the objective. They give a pretty good result.

Cross talk

Cross emission appends when the right tail of the lowest dye emission spectrum enters into the detector of the second gold when the dye emission spectrum of the lowest excited dye the highest dye (Quenching by Energy Transfer).
You create wrong co localization.
Cross excitation appends when the two excitation spectra are to close and both are emitting at the same with the same illumination
you create a wrong co localization.

The choice of secondary antibodies or dyes is essential to avoid the effects of crosstalk, in any case to avoid the crosstalk when you acquire some collocation image, you should use a method of sequential acquisition mode.
It is also very important to prepare two other samples that includes only one of the primary antibody and the two secondary antibody, one sample for each primilary antibody

We will see than what correspond of background, unspecified binding of secondary anti-bodies or pigments.
These two sample control are used determine the threshold value trough the two negative colors into the calculate the co localization

Another possibility to acquire images where the 2 stain crosstalk, the way is to acquire images in lambda mode on the two controls samples, so you can use the linear unmixture method to split the spectrum of the two stain are containing in the positive sample.

Acquisition Parameter

Gain and Offset
You should never saturate a picture. If it's the case, the image is unusable for co localization measurement.
The best picture is when you have the whole dynamical range (grey levels) in your sample.
Don't use a to high Gain value, otherwise the pmt produced some noisy image
The noise could be damaging for co localization and can produce an overestimation of the co localization. A good way to avoid this noise is to use and pmt value from 600 to 800 V if is possible, increase the Averaging number and decrease the scan speed.

Averaging
A good way to reduce the noise is to do a higher as possible Averaging during your acquisition (Confocal), depending on the bleaching of your dye. The noise is reduced by the square root of the number of averaging. It means if you have an almost noise free image with an averaging of 16, you have a pretty noisy picture with an average of 4 and an unusable picture for co localization with an average of 2.

Bit depth
The bigger the bit depth is the better dynamical range you have. It means that your information is stepped in a bigger range of gray value. Low intensity signals are well separated from high intensity signal. This information is really important for the deconvolution we should do before the final co localization.
(8 bit = 256 gray levels, 12 bits = 4096 gray levels, 16 bits = 65536 gray levels

Resolution rule and Voxel size
The objective resolution is linked with the Numerical aperture of the objective, the excitation and emission wavelength and the mounting media index refraction.

For each objective an optimal pixel size is required. If this rule is not observed you will lose a lot of details/resolution on your image.

The sampling frequency is also an important parameter which governs the resolution of the acquired image. Regarding the Nyquist theorem the smallest resolvable structure (in this case defined by the optical resolution limit) must be sampled (at least) twice in order to record all necessary information.
The maximum optical resolution resel* can be defined as the radius of the first dark fringe in the diffraction pattern, or half the diameter of the Airy disc.
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P.S. Don't forget: this is the maximal achievable theoretical (noise free system, infinite small pixels) resolution. In praxis this will never be reached! Thus following this rule you already slightly oversample.

Reference:
Colocalisation module 2007: version 1.2 J-C Sarria Biop Epfl
Co localization: McMaster Biophotonics Facility ▪ Dept. Biochemistry and Biomedical Sciences ▪ HSC 4H14, 1200 Main Street

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