• **Preparation** (Project study with Nathalie Garin)
  – Cell culture, tissue slice, special setup
  – Secondary antibody or dye choice (Cross-talk and bleed through)
  – Mounting medium, immersion medium (objective type, microscope type)
  – Control tissues for determination of Background and analysis thresholding
    (1 negative control without primary antibody and the two secondary antibody, 1x one primary antibody with both secondary antibody and 1x with the other primary antibody and both secondary for processing thresholding)

• **Acquisition** (training by José and Thierry, microscopy advices)
  – Laser alignment and chromatic aberration, control of eventual shifts
  – Optics cleaning and objective correction collar alignment
  – PMT setting (range, Laser proximity of detection – reflection)
  – Pixel size (XY resolution, Z-resolution, MAXbit acquisition for dynamical range)
  – Averaging (noise on image, statistics)
  – Gain and offset (dynamical range, background, linear acquisition, brightest condition for initial setting)
  – Sequential scanning (Cross-talk and bleed through)
  – PSF acquisition for deconvolution

• **Processing – analysis** (Training by Floyd, conception of new automated scripts)
  – Deconvolution – PSF (Huygens)
  – Object colocalization (metamorph7, Imaris5) – non Intensity dependent
  – Pixel (voxel-3D) colocalization (ImageJ, Metamorph7, Imaris5) – Intensity dependent
• **Image Types**

- *Cell cultures, tissue slides, special setups, 2D or 3D*

Fluorescence colocalization *(intensity dependent)* or object colocalization *(non intensity dependent)*
• **Preparation**
  - **Secondary antibody or dye (bleed-through and cross-talk)**

The “cross-talk” and the “bleed-through” occurs when the Emission-Excitation spectra of two dyes are too close or one of them is too wide (as DAPI).

The **cross-talk** appends when the right tail of the lowest dye emission spectrum enters the detector of the second dye (picture next page) or when the emission spectrum of the lowest dye excites the highest dye (*Quenching by Energy Transfer*). You create then a **wrong colocalization**.

The **Bleed-through** appends when the two excitation spectra are too close and both are emitting at the same time with a single illumination.

You can see here four combination dyes. The two from the top are usual combination as CFP-YFP and Alexa488 – Alexa546. Their emission-excitation spectrum are really close. **A sequential scanning and an accurate choice of detection window are roughly recommended.** The two in the bottom, Alexa488 - Alexa594 and Alexa488 – Alexa633 have a better separation avoiding most of this artifacts.

However you have to be careful if the two dyes emission are too much separate from each other, they have different resolutions and chromatic aberration (shift in Z) could appear if the objective is not corrected for this aberration. As often in science, we have to do some compromise.
• **Acquisition**

  - **Sequential scanning example with our LEICA Confocal microscope**

You can see here a typical “**cross-talk**” of the first dye going into the second detector (PMT), in turquoise. The two laser-line and the two detectors are “on” in the same time.

The only way to do a single scan for both illumination is to have dyes excitation and emission far away in the visible spectra, as you can see here.
• **Objectives**

Different type of objective are available. It is absolutely important to choose the good one 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 APOCHROMATIC objectives are corrected for blue/red/green, but ACHROMATIC objectives are only corrected for Blue/red with a little shift with the green. Each company have his own terminology.

*Ask the microscopist which objective is best suited for your experiment.*

The confocal resolution of your picture is directly linked to the NA of the objective, the wavelength (ex. and em.) and the mounting medium refractive index you’re using:

\[
\text{Lateral}_{\text{res}} = 0.51 \frac{\lambda_{\text{em}}}{\text{NA}} \quad \text{Axial}_{\text{res}} = 0.88 \frac{\lambda_{\text{ex}}}{(n^2 \sqrt{n^2-\text{NA}^2})}
\]

You have then the optimum pixel size for a given objective.

Of course, for multi-labeling you choose a mean emission-excitation wavelength

The **oil high numerical objectives** have to be use with 170 micron coverslip and are usually designate for cell culture. Their best efficiency is around maximum 20 micron after the coverslip and they have a low working distance (around 100 micron). They are the only one witch we can really do intracellular colocalization, because of they really high resolution.

The **glycerol high numerical aperture objectives** have also to be used with coverslip but they are used to scan deep tissues until 200 micron depth (if the opacity of the sample is not too high).

The little inconvenient is that we have to align the correction collar of the objective for each experiment.

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

Finally the **dry objectives** are used if it’s not possible to use the other ones. They have usually **low numerical aperture** (resolution) but **long working distance**. They can help in many situation when doing a picture seems to be impossible.
• *Resolution – Pixel size*

As told before the objective resolution is linked with the Numerical aperture of the objective, the wavelength excitation and emission 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 on your image (synthetic pictures below). A excellent automatic calculator is available on the web: [http://support.svi.nl/wiki/NyquistCalculator](http://support.svi.nl/wiki/NyquistCalculator)

Here a typical example (with synthetic image) of a wrong colocalization when the pixel size is not well suited. It could be compared with the use of a 63x/1.4 objective with a pixel size acquisition of 220nm in XY instead of 55nm. On the right 7 pixels are colocalized instead of 3 on the left, because it is not possible to separate two points of different colors close from each other. The same problem occurs if the Z distance between planes in a 3D acquisition is not perfectly calculated.
• **Other details for acquisition**

  - **Gain and Offset**
    
    You should never saturate a picture. If it’s the case, the image is unusable for colocalization. The best picture is when you have the whole dynamical range (grey levels) in your sample. When the Gain and Offset are set, don’t touch them anymore. It’s the only way we can compare or batch images.

    *If you have different conditions to compare for colocalization, always setup the Gain and Offset with the condition you know you will have the most fluorescence. This way, all the other condition will give you non-saturated signals. Each channel should have almost the same gain, to reach the full dynamical range, play with the laser intensity.*

  - **Averaging**
    
    All imaging systems produce noise (poisson distribution). This noise could be damaging for colocalization and can produce an overestimation of the colocalization. A good way to avoid this 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 colocalization with an average of 2.

    (as we will see after, the “Deconvolution” can pretty well denoise images if this is the only solution)

  - **Bit depth**
    
    The bigger the bit depth 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 colocalization.

    (8 bit = 256 gray levels, 12 bits = 4096 gray levels, 16 bits = 64000 gray levels)
• **Image processing**

Due to the optic and electronics of the acquisition system (diffraction of the light, noise induced by the electronics, the wave nature of light) the final image is relatively different from the object plane (convolution).

It is like an unwanted image processing done by the imperfection of the acquisition system. A blurred and noisy image. A part of these artifacts can be reduced.

**Filtering**

High-intensity background and noise is successfully suppressed by low pass, median, and Gaussian filters. Filtering techniques recover a voxel values from the local neighborhood and compute a weighted average. This, inherently, result in loss of resolution because the procedure convolves the image with a smoothing kernel. False-negative as well as false positive results can be generated.

A great advantage of filtering techniques is their ease of handling and their speed.

**Deconvolution** (image restoration)

Deconvolution uses the imaging properties of the optical system in the form of the point spread function (PSF) for "putting the light back where it is coming from". Imaging of an entire 3D object may be described as the convolution of this object with the PSF. Therefore, the PSF can be used for calculation of a likely model of the object from the recorded data set in an iterative process. This procedure eliminate effectively blur caused by distortion. In addition, by assuming a Poisson distribution of stray light, it suppresses background and noise to very low levels.

The technique yields images of appreciable increased contrast. This make additional objects in the low-intensity (but high-frequency!) range available to analysis. In addition, removal of noise and distortions induced by the optical system result in improved resolution, which is of critical importance in cases involving objects of near resolution size.

Disadvantage of the technique include the requirement of computing power, the exact estimation of background and \( R_{\text{inh}} \) and time.

*A separate module is given to explain and use Deconvolution at the BLoP*
• **Image Preparation**

**Region of interest (ROI)**
Not all the elements of your picture are interesting for your colocalization. It is often important to tell to the computer what is the area of interest to do the calculation.
Different methods are available to determine an ROI.
- Using a **third channel**
- Using a **segmentation method** (clustering, watershed separation, etc)
- Using **manual segmentation** with the graphic tablet

**Thresholding**
When do we consider if a low intensity pixel is signal or noise? This is the usual problem of the scientist starting a quantification from an image.

Usually, people do that **by eye, and try to keep the structure of interest** inside the thresholded pixels and throwing away what we can call “background”. But this method is not without danger assuming the user can have a predefined idea on what the result “have” to be.

A more methodical way includes the adjustment of all photomultipliers to their respective channel intensities followed by the recording of **control specimens** probed with **one primary antibody and the two secondary antibody**, for each primary antibody. We will see than what correspond of background and unspecified binding of secondary anti-bodies. The **fluorogram** (picture below) show a cloud of pixels associated with the corresponding axis. The thickness of the cloud yields the **background intercept** or threshold in the complementary axis. Processing of this data set results in **threshold value suitable for processing images**. Care must be taken not to miss the seemingly few pixels lying high above the cloud in the fluorogram.

L. Landmann, 2004
**Analysis (colocalization test)**

**colocalization Test: (facultative)**

How to know if we have effectively colocalisation or what we see a random pixel distribution? A plugin in ImageJ called “Colocalization test” can do the job. The best method is called “Costes approximation (smoothed noise)”, *(read the V. Costes paper)*

With the Costes method, the original channel 1 image is compared to 200 “scrambled” channel 2 images; the observed correlation between channel 1 and channel 2 are considered significant if they are greater than 95% of the correlation between channel 1 and scrambled channel 2.

Costes’ scrambling images are generated by randomly rearranging blocks of the channel 2 image. The size of the blocks is chosen to equal the point spread function (PSF) of the image.

<table>
<thead>
<tr>
<th>Randomisation of Ch2</th>
<th>R(obs)</th>
<th>mean±sd</th>
<th>% (R_o&gt;R_r)</th>
<th>Iterations</th>
<th>Randomisation</th>
<th>PSF width*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Costes X, Y, Z</td>
<td>0.357</td>
<td>0.002±0.003</td>
<td><strong>100%</strong></td>
<td>10</td>
<td>Costes X, Y, Z</td>
<td>0.514 μm</td>
</tr>
</tbody>
</table>

*The PSF width, is known to be 3 times the Z resolution*
Analysis

**Analysis:**

*The first question is to know if the experiment is fluorescent intensity dependent or not.*

If the experiment is **not intensity dependent** you will find in the next pages the “overlap” method and the “nearest neighbor” method for 2D analysis *(read the E. Lachmanovich paper)* and the “colocalization Channel” for 3D analysis with IMARIS.

If the experiment is **intensity dependent** you will find 2 different methods:

The **Emission method** gives you a **percentage of colocalization** based on a ratio between the total intensity of the two channels above the threshold and the intensity for both channels of the colocalized pixel above the threshold. This simple method gives precise results for most of protein colocalization quantification in cellular compartments.

The **Intensity Correlation Analysis** is based on the **Pearson’s correlation coefficient**. This method is based on Similarity between the two channel and not directly on his fluorescence. This is a statistical method witch can give a coefficient between -1 and 1 (1 is total colocalization, 0 is random colocalization, -1 is total anti-colocalization). From this correlation you can do an **Intensity correlation Analysis** if you are not only interested in the percentage of colocalization but also in the synchrony of two proteins in a complex *(read the Qi Li Paper)*
Analysis ("non-intensity dependent" colocalization)

For 2D imaging

"Overlap" Analysis:
1/ Binarization by: \( \text{top_hat}(\text{image}) = \text{image} - \text{morphological_opening}(\text{image}) \)
2/ Objects of channel 2 reduced to their center of mass
3/ Count of "Yellow Dot => percentage"
4/ Colocalization test: Channel-2 randomization (Costes & Al.)

"Nearest-Neighbour distance" analysis:
1/ and 4/ same as "Overlap" Analysis
2/ Objects of both channels reduced to their center of mass
3/ If 2 center of mass of different channels are less than a defined distance (depending on the resolution limit of the objective) they are colocalized => percentage

E. Lachmanovich, 
Journal of Microscopy, 2003
Analysis (colocalization test and "non-intensity dependent" colocalization) For 3D imaging

“Colocalization channel” by IMARIS (colocalization module)

The 3D/4D software IMARIS can calculate and generate a new channel corresponding to the colocalized voxels. The threshold for both channels is defined with control specimens (as explained 4 pages before) and a 3D object. Minimal size is set (corresponding to the biological knowledge). A ratio between colocalized objects and total objects gives a percentage of colocalisation.

www.bitplane.ch
Analysis (colocalization test and “intensity dependent” colocalization)  
For 2D and 3D

**Colocalization analysis:**

3 main formulas exist for colocalization, each one have its pros and cons.

\[
\text{Emission Method} \\
\% \text{ fluo} = \frac{\sum R_c(x_i, y_i) + \sum G_c(x_i, y_i)}{\sum R(x_i, y_i) + \sum G(x_i, y_i)} \times 100
\]

*Emission from 0 to 100%*

Interest: Visual colocalization (yellow)
Really simple interpretation
=> Take into account all pixels above a threshold

\[
\rho_o = \frac{\sum (R(x_i, y_i) - R_{avg}) \times (G(x_i, y_i) - G_{avg})}{\sqrt{\sum (R(x_i, y_i) - R_{avg})^2} \times \sqrt{\sum (G(x_i, y_i) - G_{avg})^2}}
\]

*Coefficient from -1 to 1*

Interest: Correlation of the intensity distribution between two channels
=> Similarity between shapes, not intensities

\[
\rho_o = \frac{\sum R(x_i, y_i) \times G(x_i, y_i)}{\sqrt{\sum (R(x_i, y_i))^2} \times \sqrt{\sum (G(x_i, y_i))^2}}
\]

*Coefficient from 0 to 1*

Interest: Corresponding of the 2 channels alignment
=> Take into account only pixels with both signals
Analysis *(colocalization test and “intensity dependent” colocalization)*

For 2D and 3D

**Emission method:**

\[
\% \text{ fluo} = \frac{\Sigma R_c (x_i, y_i) + \Sigma G_c (x_i, y_i)}{\Sigma R (x_i, y_i) + \Sigma G (x_i, y_i)} \times 100
\]

Emission from 0 à 100%

The threshold is set with the *control specimen*.

For each image you will have 4 lines in the Excel sheet:

1/ data for the red channel only above the th. = \(\Sigma R (x_i, y_i)\)
2/ data for the green channel only above the th. = \(\Sigma G (x_i, y_i)\)
3/ data for the red channel with green component above the th. = \(\Sigma R_c (x_i, y_i)\)
4/ data for the green channel with red component above the th. = \(\Sigma G_c (x_i, y_i)\)

Integrated = Average*Thresholded area

This calculation is done in a script with "Metamorph". (www.moleculardevices.com/pages/software/metamorph.html) Hundreds of pictures can be processed in few minutes. The script have to be modified at every new experiment to set the new thresholds.

The formula is directly set in the Excel sheet.
Analysis (colocalization test and “intensity dependent” colocalization)

Statistical Method:

\[ r_0 = \frac{\sum R(x_i, y_i) \times G(x_i, y_i)}{\sqrt{\left(\sum R(x_i, y_i)^2 \times \sum G(x_i, y_i)^2\right)}} \]

Multiply Method (Overlap coefficient)
Manders equation (Manders & al. 1993)

\[ r_0 = \frac{\sum (R(x_i, y_i) - R_{avg}) \times (G(x_i, y_i) - R_{avg})}{\sqrt{\sum (R(x_i, y_i) - R_{avg})^2 \times \sum (G(x_i, y_i) - R_{avg})^2}} \]

Covariance Method (Correlation coefficient)
Pearson equation (Gonzales and Wintz 1977)

This two methods are found in a plug-in of ImageJ Called “Intensity Correlation Analysis ICA”
http://rsb.info.nih.gov/ij/

1/ The two channels have to be separate in two images
2/ The threshold is set with control specimen
3/ define a ROI if necessary

To fully understand this analysis you should read:
Analysis (colocalization test and “intensity dependent” colocalization)

“Intensity Correlation Analysis ICA” (colocalization settings):

**Use ROI**
Choose a ROI to analyze from the drop down box. Choose either ‘none’ to analyze the whole image or “Channel 1:’ to analyze the pixels in both channels within the ROI selected in channel 1.

**Use Thresholds**
The threshold is set with *control specimen*

**Keep merged ROI**
Returns a merged image of the analyzed ROI (or full image). The merged colors are determined by the “Channel combination” selected.

**Display Color Scatter plot**
This generates a scatter-plot of red intensities vs. green intensities. The color of the scatter plot pixel represents the actual color in the image.

**Display Frequency Scatter plot**
The pixels in this scatter-plot pseudo-colored so that their color represents the frequency of the red-green pixel combination in the original image (hot colors representing high values by convention). This sort of plot contains the most information, but can be a little difficult to relate it back to the original image.

**Display intensity counts**
This generates a text window with the red intensities, green intensities and frequency of the intensity pairs. This can be exported to a spreadsheet program for further analysis.

**Current Slice Only**
Select this if you do not wish to analyze the whole stack. Only the current slice in the Channel 1 stack (and the corresponding slice in the green channel) will be analyzed.
**Analysis (colocalization test and “intensity dependent” colocalization)**

“Intensity Correlation Analysis ICA” (ICA analysis settings):

**Display ICA plots**
When checked this option draws two plots, one for the red channel one for the green.
The axes on the plots are the PDM values on the x-axis and the red or green intensity on the y-axis.
The PDM value is the Product of the Differences from the Mean, i.e. for each pixel:

\[ \text{PDM} = (\text{red intensity} - \text{mean red intensity}) \times (\text{green intensity} - \text{mean green intensity}) \]

**Crosshair size (pixels)**
The points of the ICA plots are plotted as cross-hairs. You can select the size of the crosshair here.

**Display PDM image**
This option generates a new image where each pixel is equal to the PDM value at that location. The Image is pseudo-colored and a PDM scale bar is inserted.
For clarity, pixels that are below average in both channels are excluded.

**Display +ves only**
This option will generate a 2 slice stack. The first images pixels are the positive PDM values resulting from both pixels above the mean (i.e. red intensity-mean red intensity and green intensity-mean green intensity are both positive). The second slice are pixels that have pixel values in each channel which are both below the mean (i.e. red intensity-mean red intensity and green intensity-mean green intensity are both negative).

**List PDM values**
This generates a list of PDM values that can be exported to spreadsheet programs for further
Analysis (colocalization test and "intensity dependent" colocalization)

"Intensity Correlation Analysis ICA" (Result window):

Image
The name of the images and the ROI that has been analyzed is entered in the Image column. If no ROI was selected or the "Use ROI" option unchecked then the ROI describes the image dimensions.

Rr
This is the Pearson’s correlation coefficient. Zero-zero pixels are not included in this calculation. This is a popular method of quantifying correlation in many fields of research from psychology to economics. In many forms of correlation analysis the values for Pearson’s will range from 1 to -1. A value of 1 represents perfect correlation; -1 represents perfect exclusion and zero represents random localization. However, this is not the case for images. While perfect correlation gives a value of 1, perfect exclusion does not give a value of -1. Low (close to zero) and negative values for Pearson’s correlation coefficient for fluorescent images can be difficult to interpret. However, a value close to 1 does indicate reliable colocalization.

R
This is Mander’s Overlap coefficient. This is easier than the Pearson’s coefficient to comprehend. It ranges between 1 and zero with 1 being high-colocalization, zero being low. However, the number of objects in both channel of the image has to be more or less equal.

Ch1:Ch2
This value represents the “red: green” pixel ratio. The Overlap coefficient (R) is strongly influenced by the ratio of red to green pixels and should only be used if you have roughly equal numbers of red and green pixels (i.e. Nred / Ngreen ≈ 1).

M1 and M2
These split coefficients are Mander’s Colocalization coefficients for channel 1 (M1) and channel 2 (M2). These split-coefficients avoid issues relating to absolute intensities of the signal, since they are normalized against total pixel intensity. We also get information as to how well each channel overlaps the other. There are cases where red may overlap significantly with green, but most of the may not overlap with the red.

If the assumption is made that greyscale number equates to dye molecules (this is not necessarily correct) then these coefficients represent the percentage of red dye molecules that share their location with a green dye molecule. These coefficients are very sensitive to poor background correction and do not take into account the intensity of the second channel, other than it is non-zero. For example, a bright red pixel colocalizing with a faint green pixel is considered equivalent to a bright red pixel colocalizing with a bright green pixel. Intuitively, a red-green pixel-pair of similar intensities should be considered “more colocalized” than a pixel pair of widely differing intensities.

<table>
<thead>
<tr>
<th>Results</th>
<th>Rr</th>
<th>R</th>
<th>ch1 ch2</th>
<th>M1</th>
<th>M2</th>
<th>N+R0</th>
<th>N+G0</th>
<th>ICQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Image</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ch1:3f and Ch2:3f x 0 y0 z1 w245 h251</td>
<td>0.566</td>
<td>0.511</td>
<td>0.505</td>
<td>1.000</td>
<td>0.569</td>
<td>45533</td>
<td>61473</td>
<td>0.256</td>
</tr>
<tr>
<td>Ch1:3f and Ch2:3f x 10 y1 48 z1 w51 h46</td>
<td>0.790</td>
<td>0.945</td>
<td>0.708</td>
<td>1.000</td>
<td>0.876</td>
<td>1532</td>
<td>2345</td>
<td>0.291</td>
</tr>
<tr>
<td>Ch1:3f and Ch2:3f x 50 y90 z1 w107 h96</td>
<td>0.806</td>
<td>0.760</td>
<td>1.144</td>
<td>0.978</td>
<td>0.977</td>
<td>5987</td>
<td>7858</td>
<td>0.282</td>
</tr>
</tbody>
</table>
Analysis (colocalization test and “intensity dependent” colocalization)

“Intensity Correlation Analysis ICA” (Result window):

N+ve
This represents the number of pixel pairs that have a positive PDM value

Ntotal
This is the number of pixels pairs in the images that where at least one of the pixel pairs is above zero

ICQ
This is the Intensity Correlation Quotient.
If the intensities in two images vary in synchrony (i.e. they are dependent),
they will vary around their respective mean image intensities together. 
So, if a pixel’s intensity is below average in the red channel (i.e. Ri-Rmean< 0);
it will be below average in the green channel (i.e. Gi-Gmean<0).
Similarly, if a pixel is above average in one channel it will be above average in the other. Therefore, in an image where the intensities vary together, the product of the differences from the mean (PDM), will be positive.
The converse is true. If the pixel intensities vary asynchronously, i.e. 
the channels are segregated so that when a red pixel is above average, 
the corresponding green pixel is below average; then most of the PDMs
will be negative.

The ICQ is based on the non-parametric sign-test analysis of the PDM values and is equal to the ratio of the number of positive PDM values to the total number of pixel values. The ICQ values are distributed between -0.5 and +0.5 by subtracting 0.5 from this ratio.

Random staining: ICQ~0; Segregated staining: 0> ICQ³ -0.5;
Dependent staining: 0<ICQ£+0.5

PDM is equal to the value (A-a)*(B-b) as described in Li et al. 2004
Analysis ("intensity dependent" colocalization)

For 3D imaging

Main window of the colocalisation module in IMARIS

After setting the thresholds we can have directly preliminary results in the "Analysis result" window.
**Analysis** ("intensity dependent" colocalization)

For 3D imaging

The results you see in the main window are approximate results. If you need an accurate result taking in account all the voxels you have to click on the **"build coloc channel"** to make a channel with the voxels which have a value above the threshold for both colors.

And then click on **"Channel Statistics"** to have all the data concerning the colocalisation channel.

You can then export the data in **"CSV"** format, which can be read by a tabloid.
Bibliography


A syntaxin 1, G-alpha0, and N-Type Calcium Channel Complex at a Presynaptic Nerve Terminal: analysis by Quantitative Immunocolocalization – Qi Li and al. The Journal of Neuroscience, April 21, 2004 – 24(16): 4070-4081


