Protein concentration quantification in tissues immunolabeled for extracellular matrix components (G. Rubí-Sans & E. Rebollo)

This imaging protocol is aimed to quantify the relative concentration of different extracellular matrix (ECM) components in immunolabeled tissue samples. For each ECM protein of interest, a first calibration step is carried out, where increasing concentrations of cross-linked protein drops are immobilized onto a nitrocellulose membrane, immunostained and imaged. Their intensity quantification is used to address the linear correlation between intensity and protein content; the linear regression slope is then used to convert intensities into protein amounts in the target tissue regions analyzed, which should be imaged under the exact same illumination conditions. Last, protein amounts are normalized by area so that the concentrations of the different ECM components analyzed can be directly compared. This approach provides detailed ECM composition information in studies of tissue morphogenesis, regeneration, and disease progression. The complete protocol and applications are described in the article *Quantification of extracellular matrix components in immunolabeled tissue samples (Rubi-Sans G, Cler M, Vall-Lacalle M, Nyga A, Pérez-Amodio S, Rebollo E, Mateos-Timoneda MA, Rebollo E and Engel E; Submitted to Nature protocols)*. This note explains how to use the macros *Protein Content Calibration.ijm* and *ECM Protein Content Measurement.ijm*, developed for intensity calibration and tissue ECM quantification respectively, and explains the image processing pipelines, so that they can be easily adapted to other similar protocols.

A. Calibration procedure.

The following pipeline must be applied individually to each calibration dot.

How to use:

1. Open the image containing the protein fluorescent dot. It must be open as multidimensional xyz hyperstack. Fiji can handle big images created as a mosaic of merged 3D tiles.
2. Open the macro *Protein Content Calibration.ijm* by drag and drop to the Fiji bar and hit *Run*.
3. When prompted, draw a ROI on the active image to select the reference background region.
4. When prompted, draw a polygon around the fluorescent dot.
5. The macro will now run and deliver a *Log* file containing the image name and the *Raw Intensity density* of the selected region.

Imaging pipeline:

The macro performs all the following steps:

1. **Image projection:** The original stack is automatically Z-projected (using the maximum projection).
2. **Background subtraction:** The macro asks the user to select a region of interest (ROI) outside the fluorescent signal (figure below, left panel); the purpose of this ROI is to be used as reference of the background generated by the membrane where the drop has been seeded and the mounting conditions. The macro automatically subtracts the mean value of the background ROI. The resulting image (figure below, right panel) will be used for the final intensity measurements.

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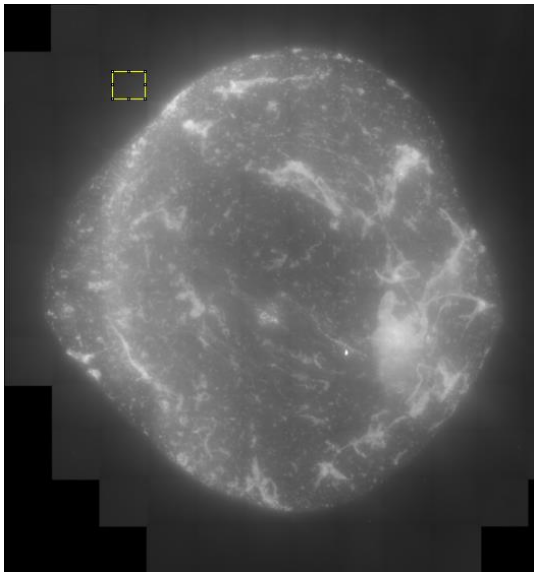
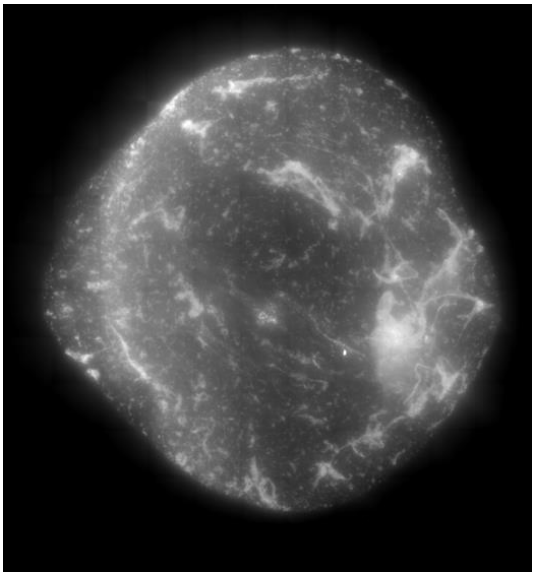
Maximum projection

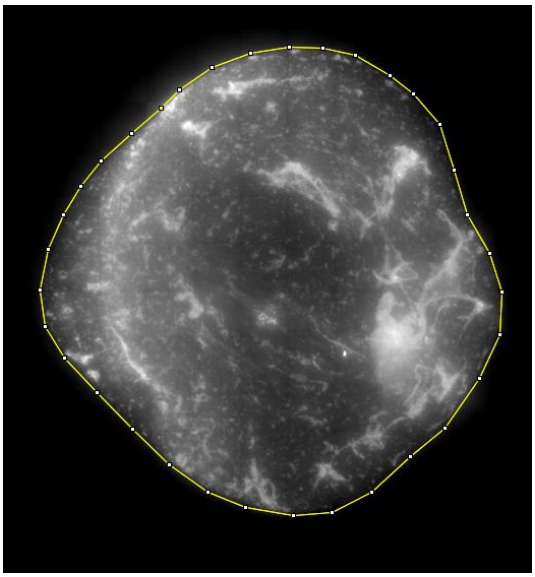


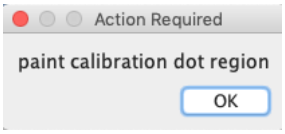
Background subtracted



3. **Manual area selection:** The macro asks the user to draw a polygon line around the edge of the fluorescent signal; this ROI will be used later for final measurements.

Manual area selection





4. **Automatic segmentation:** The whole image is duplicated and automatically segmented to create the binary mask. To this purpose, the contrast is enhanced by applying a local contrast algorithm; then, the background is homogenized using the rolling ball algorithm (radius 20), and the median filter (radius 1) is used to further eliminate the noise. The *Otsu* thresholding method is then applied to generate the binary mask.

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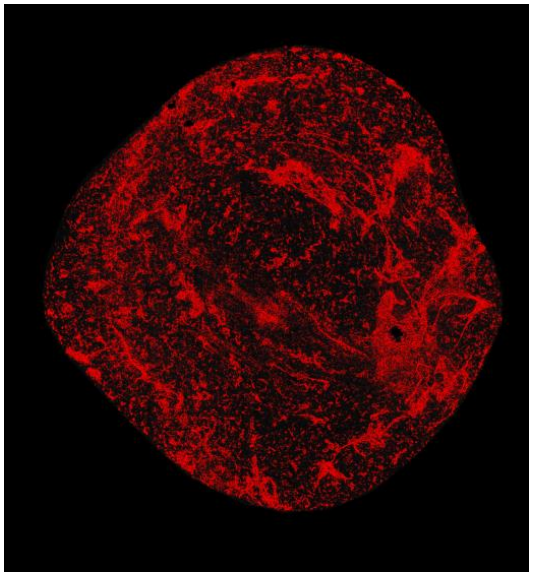
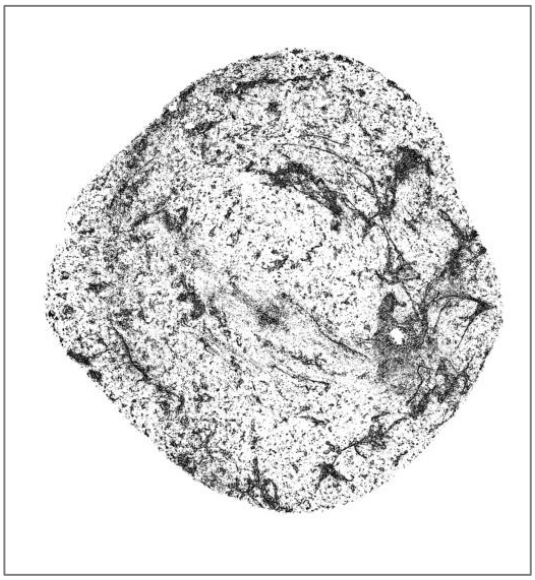
Thresholding

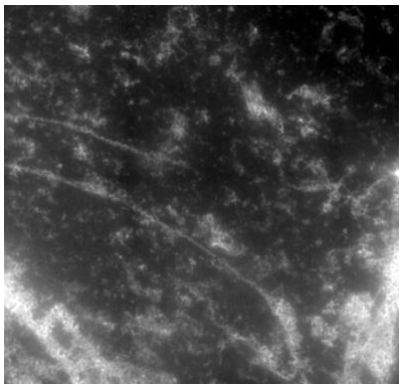


Binary mask

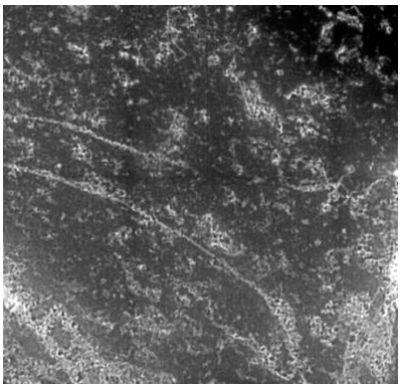


5. The images below show a detail of the complete segmentation pipeline on a smaller image region.

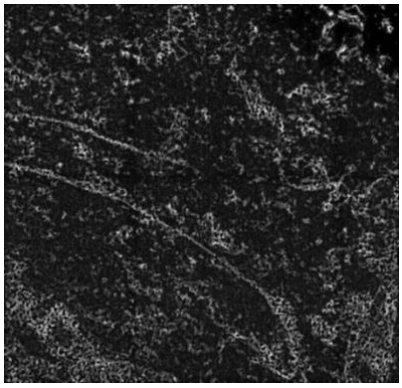
Image region



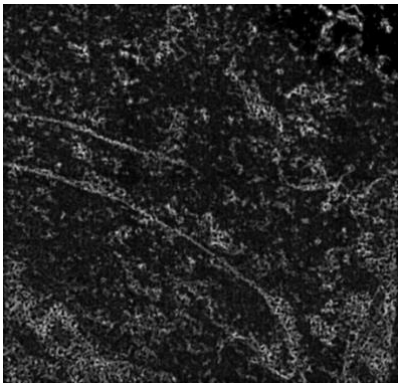
Enhance local contrast



Subtract background



Median filter



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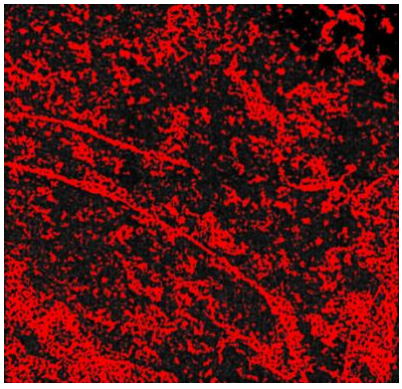
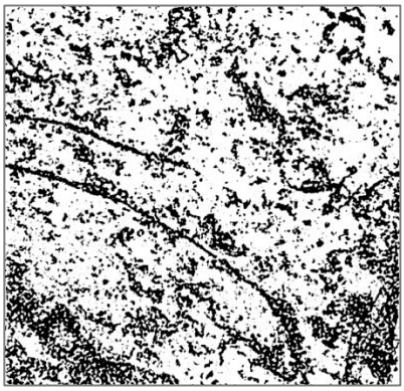
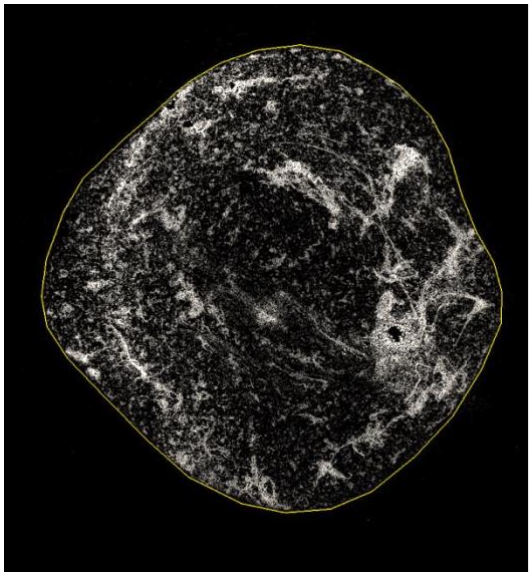
Otsu threshold Binary mask





6. **Create selection:** All positive (255) pixels within the created mask are converted into a single selection and added to the ROI Manager. This ROI will be used to measure the *Raw Intensity Density* (the sum of the intensity of all the pixels) on the fluorescence image delivered in step 2 (projected and background subtracted fluorescence image).



Results

	Area	Mean	IntDen	RawIntDen
1	639041.09	18.63	11907785.71	1118805185

B. Quantification procedure.

Before running this procedure, the slope values of the calibration curves should have been obtained for each of the ECM components to quantified step (A). Also, the BG average levels should be manually calculated for each channel of the images to be analyzed. This can be easily done by:

1. Open a target image by drag and drop to the Fiji bar.
2. Go to *Image>Stacks>Z Project…*, choose Max Intensity and hit Ok.
3. Go to *Image>Color>Make Composite*.
4. In *Image>Adjust>Brightness/Contrast…*, hit *Auto*, so that the dark areas can be easily identified.

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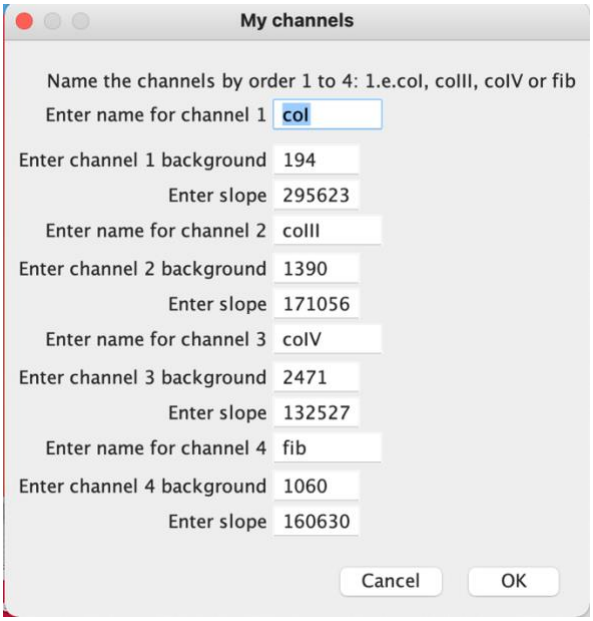
5. Draw a small square within a background region and add it to the *ROI Manager* by pressing "t" on the keyboard.
6. Go to *Analyze>Set Measurements*… and choose *Mean gray value*, click ok.
7. On the *ROI Manager* window, select the ROI and click measure. Repeat this operation for each of the channels in the composite (change from one channel to another using the image lower bar).
8. Repeat the calculations on several images, the average background values obtained will be then introduced in the general automated macro (next step).

How to use:

1. Open the macro named *ECM Protein Content Measurement.ijm* by drag and drop to the Fiji bar and hit *Run*.
2. A browser will pop up for the user to select the origin folder containing the images to be quantified. They must be multidimensional images created as xycz hyperstacks in *.tif format.
3. Next, a new browser will pop up for the user to select the destination folder where the results table and the verification images will be saved.
4. Then, a dialog box will show up, asking for names to identify channels C1 to C4. For each channel, the calibration curved parameters (slope and independent term, previously estimated during the calibration step) and the background level, must be introduced.



5. The macro will now sequentially process all the images from the origin folder and deliver to the destination folder a text file containing the protein concentrations per channel and per image, plus a list of verification images that can be used to track back any possible outlier result.

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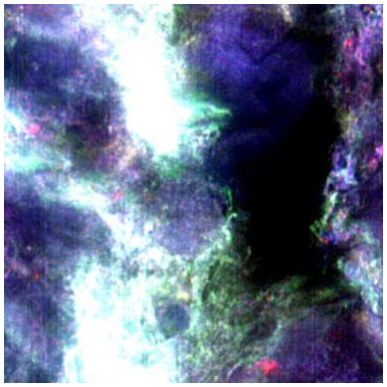
Imaging pipeline:

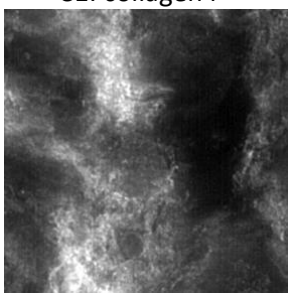
For each image, the macro performs automatically all the following steps:

1. **Image preparation:** the original stack is Z-projected (using the maximum projection), and the channels are split and renamed according to the user indications (this enables using any arbitrary channels order during image acquisition).

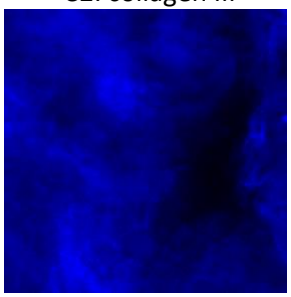
Maximum projection



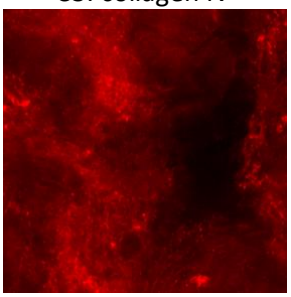
C1: collagen I



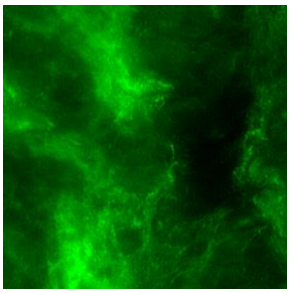
C2: collagen III



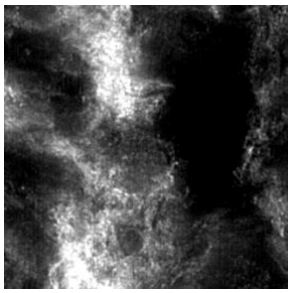
C3: collagen IV

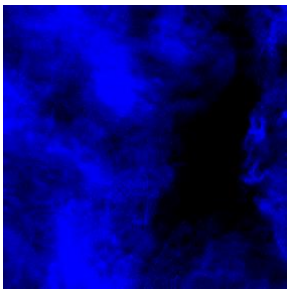


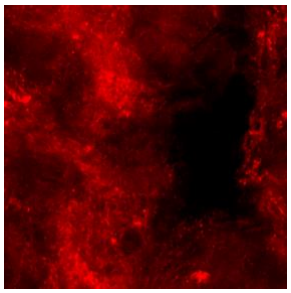
C4: fibronectin

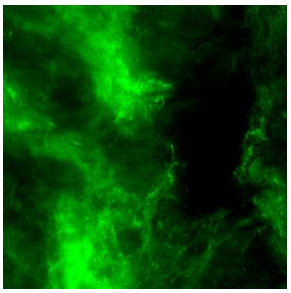


2. **Background removal:** The background mean value introduced by the user in the first dialog box is automatically subtracted from each channel. The resulting image channels (figure below) will be used for the final intensity measurements.









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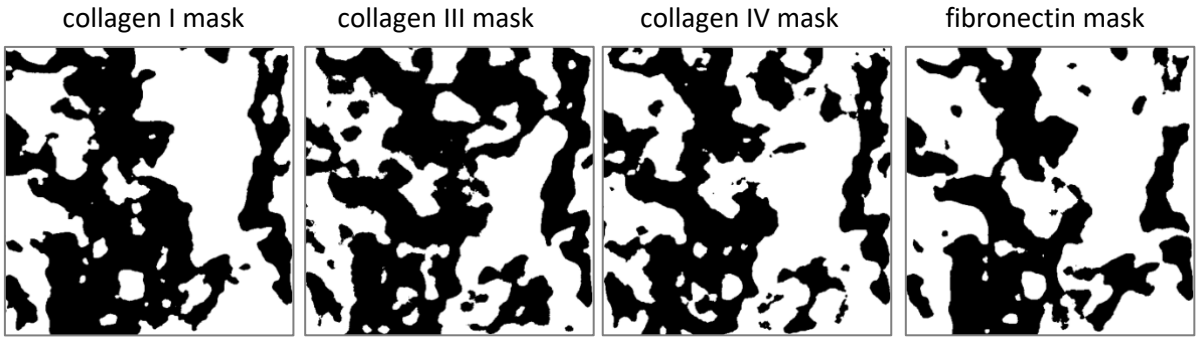
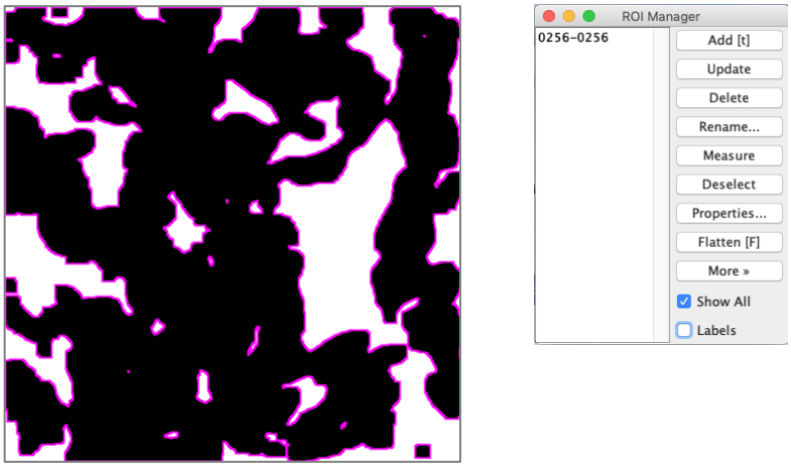
3. **Image segmentation**: For each independent channel, a mask is created, containing all possible labelled structures. Local contrast enhancement is used to avoid leaving low intensity regions unmasked. Median filtering is used to prepare the image for general automatic thresholding. The idea behind is to make the segmentation procedure as permissive as possible, to avoid segmentation biases due to particularly strong structures. Finally, general automatic thresholding is applied (Huang method) and the image is converted into a binary mask. All these parameters can be easily modified in the code to adapt the segmentation pipeline to different types of labeling.



4. **Region of Interest (ROI) selection:** The four independent masks are summed into one; the resulting positive pixels conform the ROI that is loaded onto the *ROI Manager* to be used for measurements.



5. **Intensity and Area measurements:** Within the ROI, the *Raw Intensity Density* is measured on each individual channel that resulted from step 2; each obtained value corresponds to the "y" term of the line equation y = ax, and "a" corresponds to the slope of the calibration curve previously estimated for each protein. The area of the ROI is also measured so that protein contents can be normalized by area and compared between the different samples

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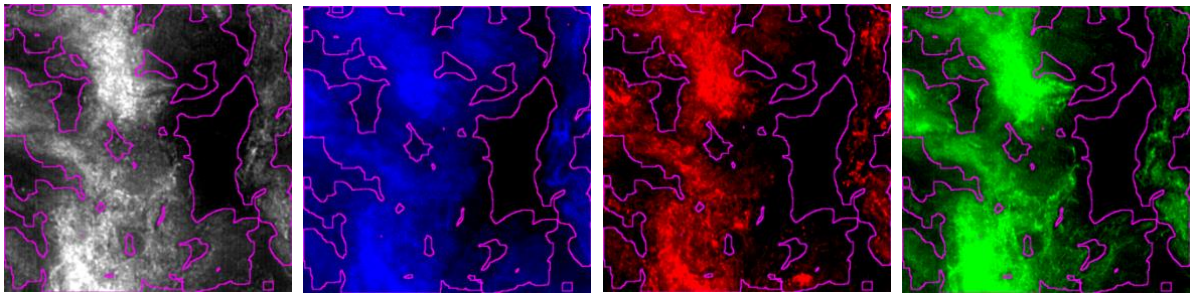
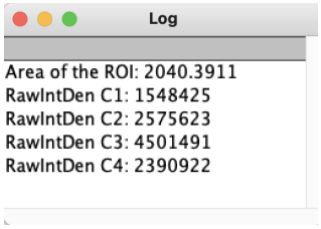
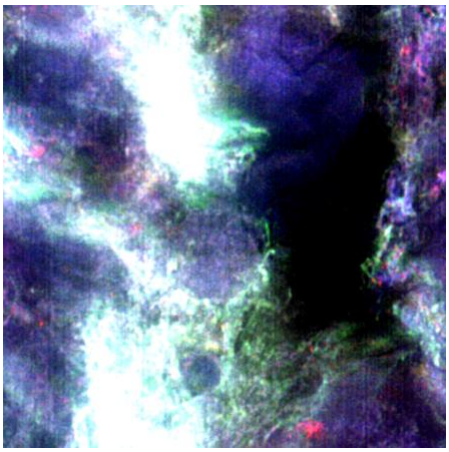




6. **Values conversion:** The macro automatically converts all raw intensity values into protein content (in mg) by clearing the "x" term of the line equation as x = y/a, and delivers a data sheet containing al the protein contents and areas measured for each image.
7. **Verification image creation:** For each image analyzed, a 4 channels composite is created where the ROI selection is overlaid. Such image is saved as an RGB for further verification of the segmentation procedure.

Original Z-proyected image



Verification image