

An ImageJ workflow for automated laser capture microdissection of subcellular structures



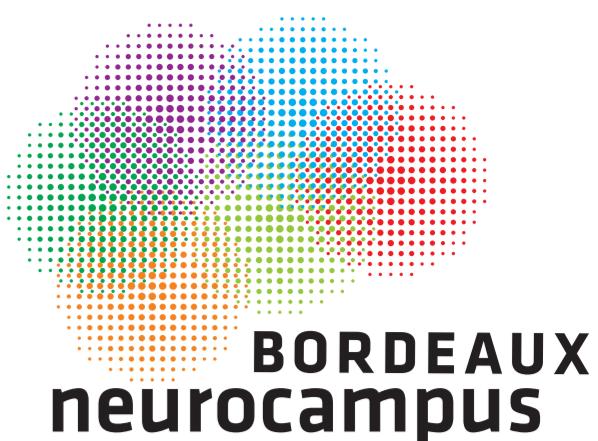
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Abstract

When trying to combine highly demanding techniques such as laser-microdissection and proteomics, one would come across two types of difficulties: 1-the required user input to delineate and isolate structures of interest; 2-quantities of material required to faithfully analyse the protein content of samples.

Invadosomes are actin rich structures involved in the degradation of extracellular matrix (ECM), present both in physiological and pathological cells. Invadosomes can take different shapes such as rosettes (5-10 µm of diameter). Deciphering their composition is of main relevance to understand the interplay between cells and their ECM during migration and invasion. We first started isolating invadosomes using laser-microdissection, manually delineating individual structures. On average, a trained user is able to isolate up to 200 structures per hour, while a goal of 40000 should be reached for proper proteomic analysis.

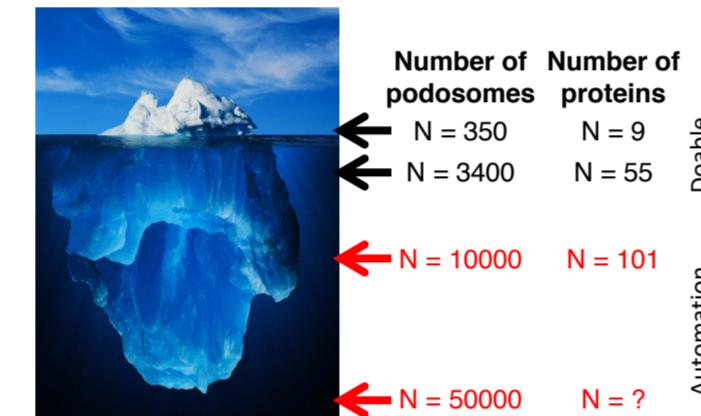
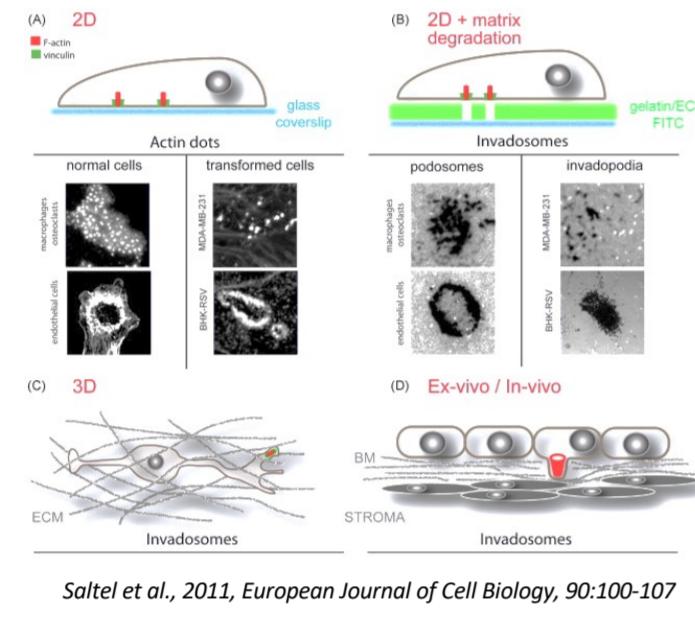
Actin-targeted labelling of invadosomes allows a non-equivocal identification of the structures. Their geometry, a donut-like shape, is well-defined, and make them easily retrievable from an image. We therefore decided to automate the detection, trying to feed the laser capture microdissection (LCM) driving software with the detected regions of interest (ROIs).

Unfortunately, the Zeiss PALM system we use is not provided with an API: a direct implementation of third-party functionalities is impossible. We circumvented this drawback by writing an ImageJ workflow, made of two parts: a plugin converting ROI Manager stored delineations into "elements" file (the input ROI format for the Zeiss device); and a toolset performing the actual detection.

The full automation is made in two steps. First the sample is scanned by the LCM device, collecting individual images of each visited field. As images are saved to the disk, the workflow will automatically process them. ROIs are extracted, and converted into the "elements" files using the plugin. In a second step, the user has to push this file to the LCM software, relax, and let the system do the job! Using this semi-automated optimized workflow, we routinely successfully were able to isolate 900 structures per hour, on average.

This proof-of-concept study was performed on invadosomes, but could well be used for any kind of detectable structures. Next version of the toolset will broaden its application domain. It will include a functionality aimed at the creation of a detection profiles system, providing the user with a way to create, edit and re-use detection workflows.

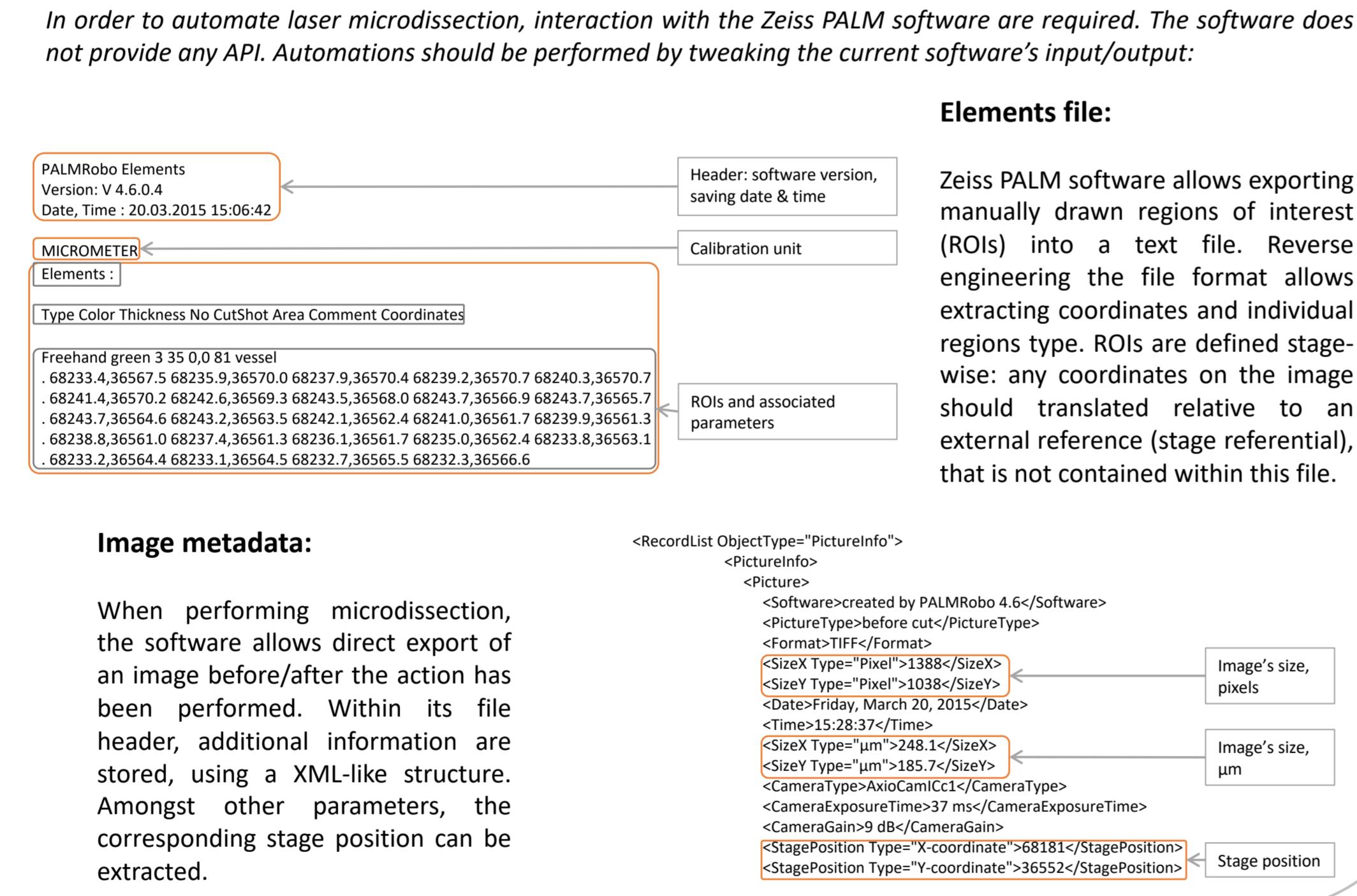
Biological background and methodology



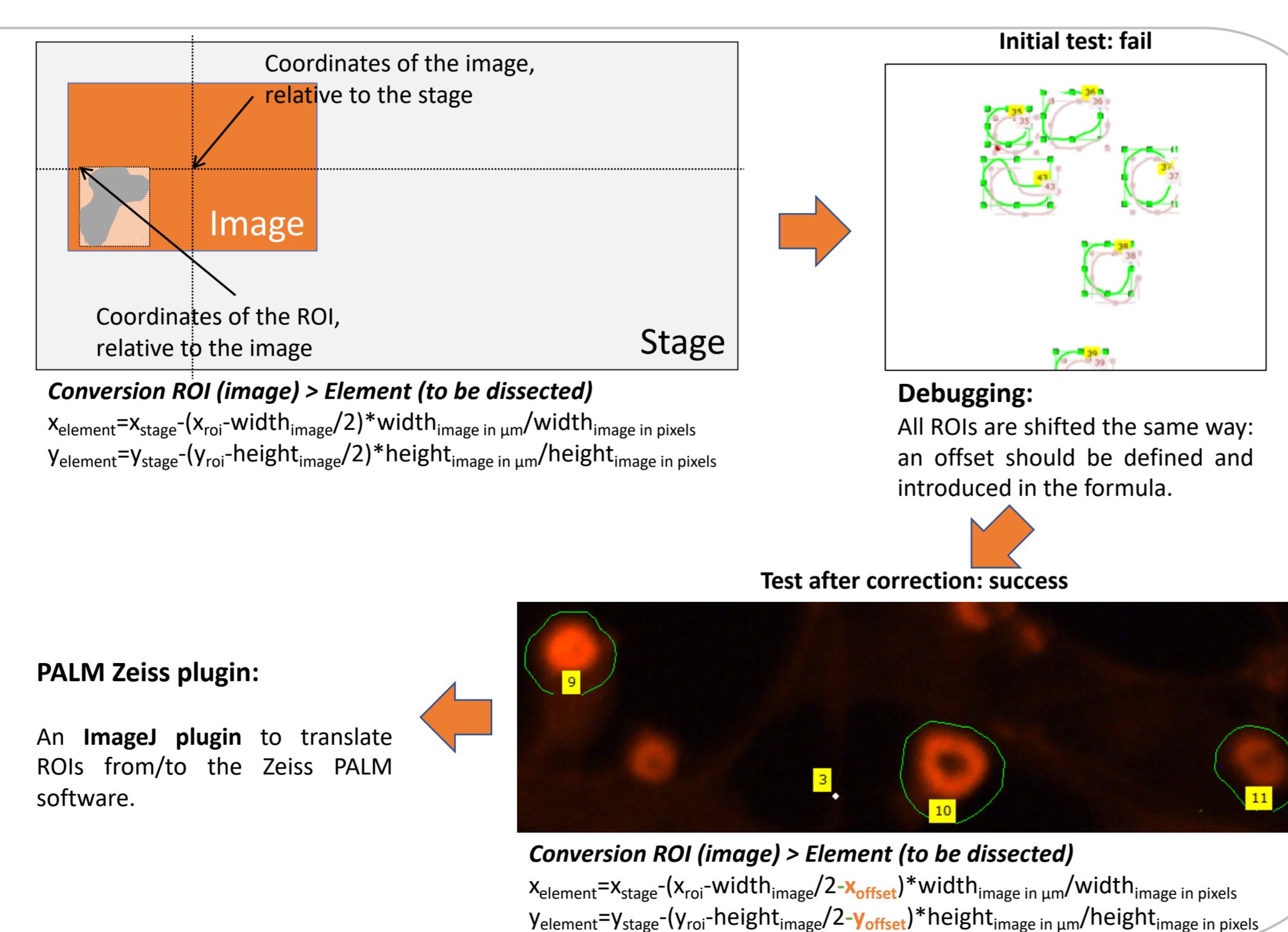
Laser microdissection allows isolation of individual structures based on user-defined regions of interest. Two strategies exist where the dissected structure is either collected by gravity or catapulted towards a proper container. In this study, we used the Zeiss PALM catapulting-based system.

Once isolated, the proteic composition of structures is determined by mass spectrometry. Identifying low abundance peptides highly depend on the quantity of collected material. On our first attempts 55 unique proteins were identified out of 3400 structures. Our goals is to identify as many invadosomes constituents as possible.

What kind of information can we use?

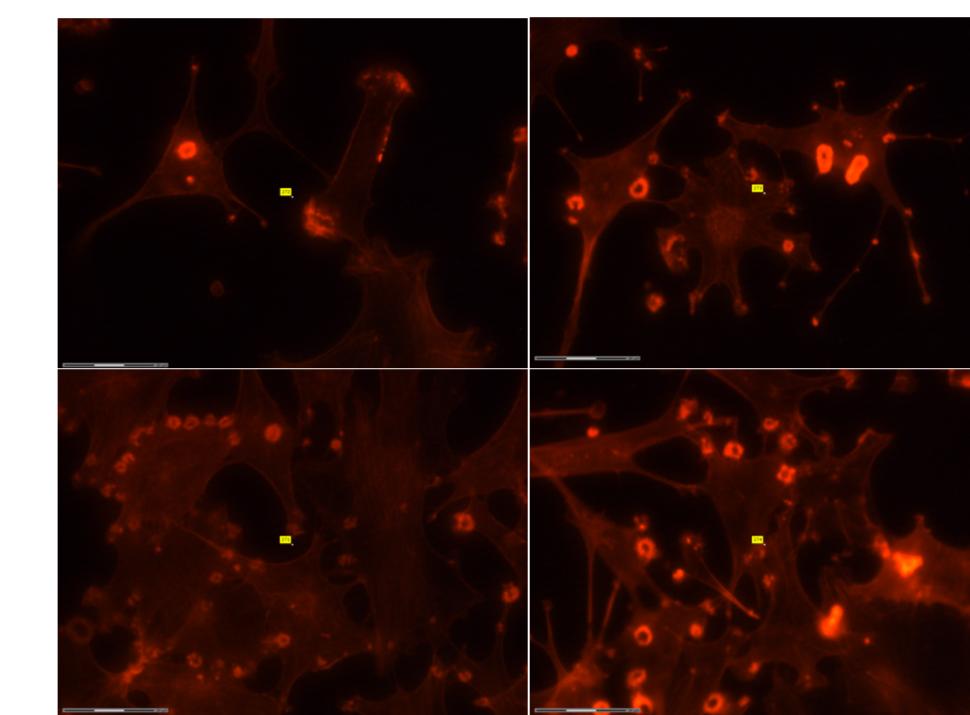


Understanding the ZEISS PALM coordinates system



Building an image-based detection workflow

Invadosomes are actin-rich, round-shaped structures. In our experiments, they are labelled using the Life-Act actin construct. Those two characteristics make them quite easy to distinguish by fluorescence microscopy, although the detector used on the Zeiss PALM system is a low sensitivity color camera. The workflow used for structures' delineation is quite simple, although it has been designed to be robust enough in order to achieve reproducible results:

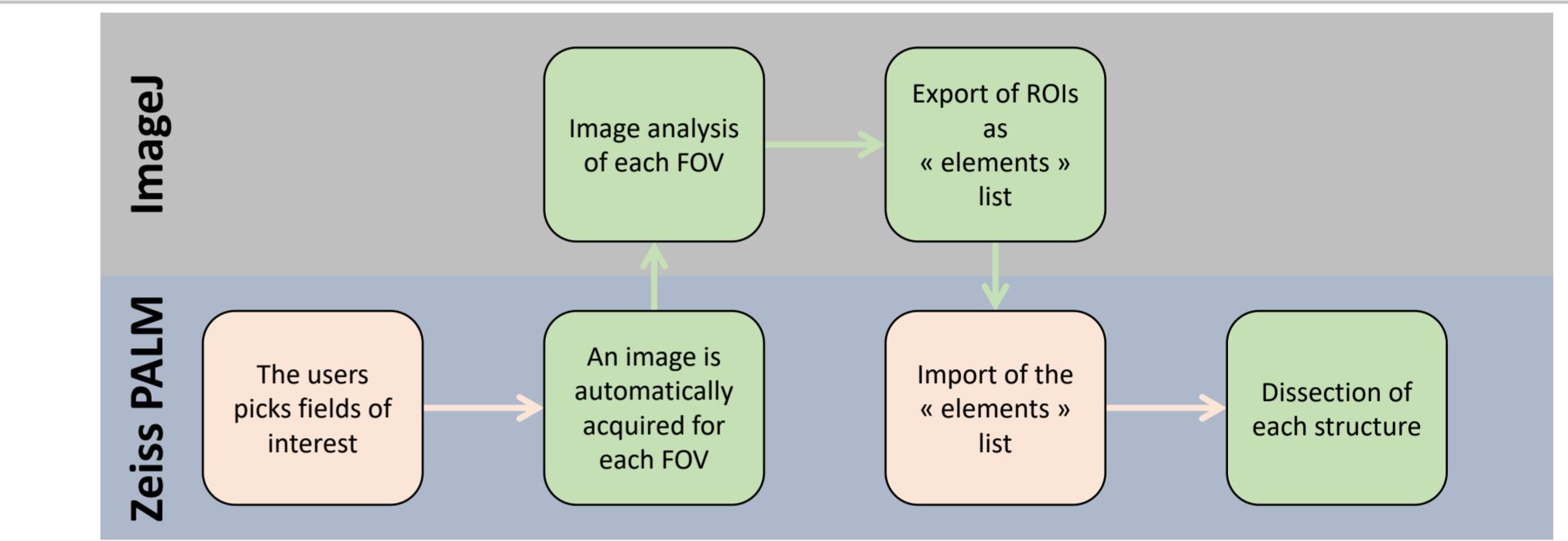


- Extract red channel
- Remove the scale bar
- Median filter, radius: 2 pixels
- Subtract background, rolling ball, radius: 2 pixels
- Automatic threshold, default method
- Binary mask
- Morphological closing (4 iterations)
- Fill holes
- Connexity analysis (size filter >1000 pixels, circularity >0.35)
- Dilation of each single ROI by 15 pixels

The detection workflow is written as an **ImageJ macro**. It can be adapted and generalised to any kind of structure. Once detection has been performed, ROIs are pushed to the ROI Manager. The Zeiss PALM plugin is then called to translate ImageJ ROIs into Zeiss PALM Elements.

This workflow can be adapted and generalised to any kind of structure.

Building an integrated workflow, version 1



The workflow is written as an **ImageJ toolbar**. The full process is performed in 3 steps:



Button 1: Set Parameters
Setup parameters such as offset, number of images to analyse etc...



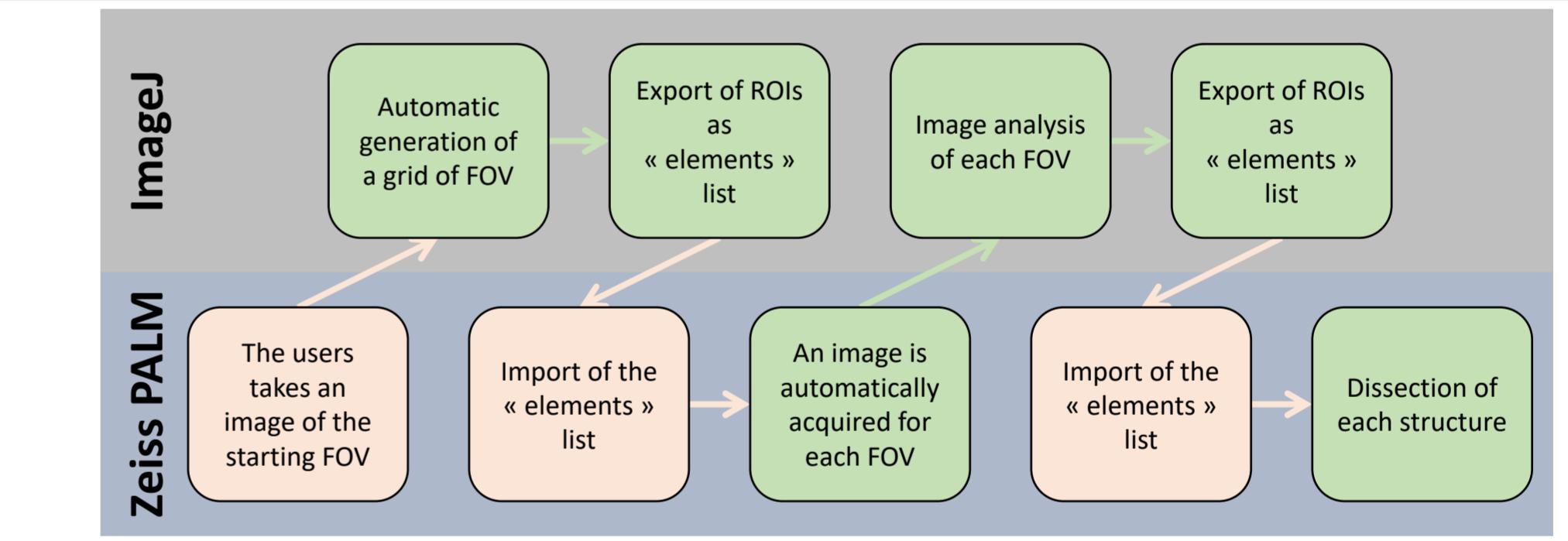
Button 2: Monitor Folder
Defines the target directory where images are saved by the Zeiss PALM software and starts monitoring the folder for new images. Each time a new image is saved, it is automatically analysed, « elements » file is saved and pulled to the main « elements » list.



Button 3: Stop Monitoring Folder
Ends looking for new images

On average, 450 structures can be isolated per hour
(gain >x2 as compared to manual processing)

Building an integrated workflow, version 2



The workflow, version 2, is also written as an **ImageJ toolbar**. The full process is performed in 6 steps:

Button 1: Full Process

Starts the procedure, fully automated

Button 3: Create Folders

Generates a normalised data structure: creates folders to store images and « elements ».

Button 4: Make Grid

Generates the acquisition grid from the stage coordinates stored in the active image.



On average, 900 structures can be isolated per hour
(gain >x4 as compared to manual processing, reduced field selection bias)

Conclusions and future work

New insights on invadosomes composition

Our method allowed us to selectively isolate more than 40000 invadosomes. After mass spectrometry, 570 proteins were identified with ≥1 peptide, 366 proteins identified with ≥2 peptides.

Thanks to our approach, we highlighted and demonstrated for the first time an internal protein translational activity into the invadosomes (manuscript under revision). Notably, some mRNA binding proteins identified with our methodology are already associated with cancer progression and invasion.

In the future, this new invadosome feature could pave the way for the identification of a translation signature for tumor cell invasion, which could be then targeted pharmacologically.

Ongoing work

The workflow presented here has been specifically aimed at isolating one kind of structure. We are currently working on reshaping the toolbar so that user profile can be designed, saved and call-back in order to account for each sample's specificity.

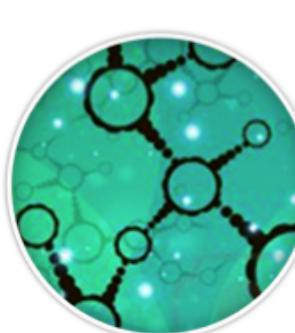
Extending the workflow's potential

One axis for our ongoing developments is to provide tools coupling digital pathology, laser microdissection and mass-spectrometry.

We are currently developing and testing a new tool, aimed at correlating positional data from slide scanners to laser microdissection systems. The former would be used for batch imaging. Images are made available remotely to the pathologist for annotation. Finally, drawn ROIs are translated into a laser microdissection compatible format for isolation before mass-spectrometry analysis.



The full workflow, toolbar and plugin, is available on GitHub: https://github.com/fabricecordelieres/IU_PALM_Zeiss_workflow



FRANCE-BIOIMAGING



PLATE FORME DE MICRODISSECTION