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Image Intensity Processing and Analysis with ImagePro and Prism

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Lexi Simar¹, Andrew Barlow², Andrew Molnar², Eduardo Rosa-Molinar PhD^{1,3}

¹The University of Kansas; ²Media Cybernetics;

³The Washington University in Saint Louis School of Medicine, Washington University Center for Cellular Imaging



Lexi Simar

Columbia University

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We use this protocol and it's working

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Abstract

Image processing and analysis completed with the use of ImagePro and GraphPad: Prism. The intensity values are exported through evaluation of the raw and deconvoluted images through ImagePro. The values are then analyzed through Prism via a two-way repeated measures ANOVA.

Guidelines

This analysis can be run on single images as well as z-stacks. A consistent ROI and n value should be used across each group of the analysis if the repeated measures ANOVA is being used.

Materials

Software:

1. ImagePro (Media Cybernetics, current version 11.1, build 10023)
2. Slidebook (Intelligent Imaging Incorporated, version 2024 2.0.45035)
3. Prism 10 (GraphPad, current version 10.4.1 (627))

Before start

To run images in ImagePro that were not obtained through this software, the file needs to be an OME tiff format. You can run individual channels at a single time through separate files (_C1 vs _C2) or run all channels at the same time selectively (_C0). File naming is explained in the protocol.

Image Processing: ImagePro

- 1 Convert image files into 16-bit OME Tiff files.



Note

Z-stack or single plane images can be converted.

- 1.1 If images were obtained through Slidebook, export the images as OME Tiff and check the XML document option in the export window.

- 2 Open ImagePro software.

- 3 Open additional packages if needed; this can include a macro script that will print out the PSF of the image after the deconvolution is run.

- 4 Open initial OME tiff image with notation C0 at the end of the file name (if using Slidebook to convert). This notation includes all channels in the image file, unlike C1 or C2.

- 5 On the right side of the image, adjust each channel to correspond to the label/fluorophore of your image.

Note

This can also correspond to what your PMTs are labelled for in two-photon imaging, if looking at autofluorescent signals.

- 6 In the Process ribbon, open the **AutoQuant Deconvolution** function.

AutoQuant Deconvolution

1. Confirm Image Metadata

Active Image

20_NewMix_Cygel_dual_850_1024_S

Volume Dimensions

X: 1080, Y: 216, Z: 1080

Locations

2 (2 channels)

Calibrations

X: 0.359µm Y: 0.359µm Z: 0.3...

Objective Lens

W "Plan-Apochromat" 20x/1.0

Lens Immersion RI

1.333

NA

1

2. Confirm Channel Metadata

Channel	Modality	λ (nm)	
☒ 1: PMT 2 - Pol 2	Two Photon	590	
☒ 2: PMT 3 - Pol 2	Two Photon	508	

3. Choose Starting PSF

☒ Theoretical PSF

☐ Derived PSF

☐ Measured PSF

4. Choose Deconvolution Settings

☒ Use Defaults

Settings

Deconvolve

Figure 1. AutoQuant Deconvolution sidebar window that opens for the user to provide all values and information needed to run a successful deconvolution.

6.1

Note

This needs to be done only once per sample type or objective type.

- 6.2 In "2: Confirm Channel Metadata" fill in the boxes for objective lens, immersion RI, and NA values.

Note

This can be populated through the objective drop down menu if the information has already been tied to the objective and saved within the program.

- 6.3 In "2: Confirm Channel Metadata," select each channel or label, choose the instrument modality from the drop-down menu, and confirm the emission wavelength for that channel.

- 6.4 If all channels do not need to be processed for the deconvolution, deselect the box to the left of the channel name for the unwanted channels.

This can also be done below the image in the channels bar (above z plane bar). Use the purple triangle to "crop out" or exclude the channels not wanted in the deconvolution by moving them left or right.

Note

If the channel is unselected, the deconvolution will take longer than necessary as the channel remains visible to the process; thus, excluding it through the channel bar below the image is preferred.

- 6.5 In "4: Deconvolution Settings," select "default" settings (default can be changed through the settings button) and the theoretical PSF option selected in "3: Choose Starting PSF."

Note

If you have a PSF of a bead for the channel or the system this can be used as well. Double check the iteration value needed; for this pollen experiment 20 iterations were used (default is 10).

6.6

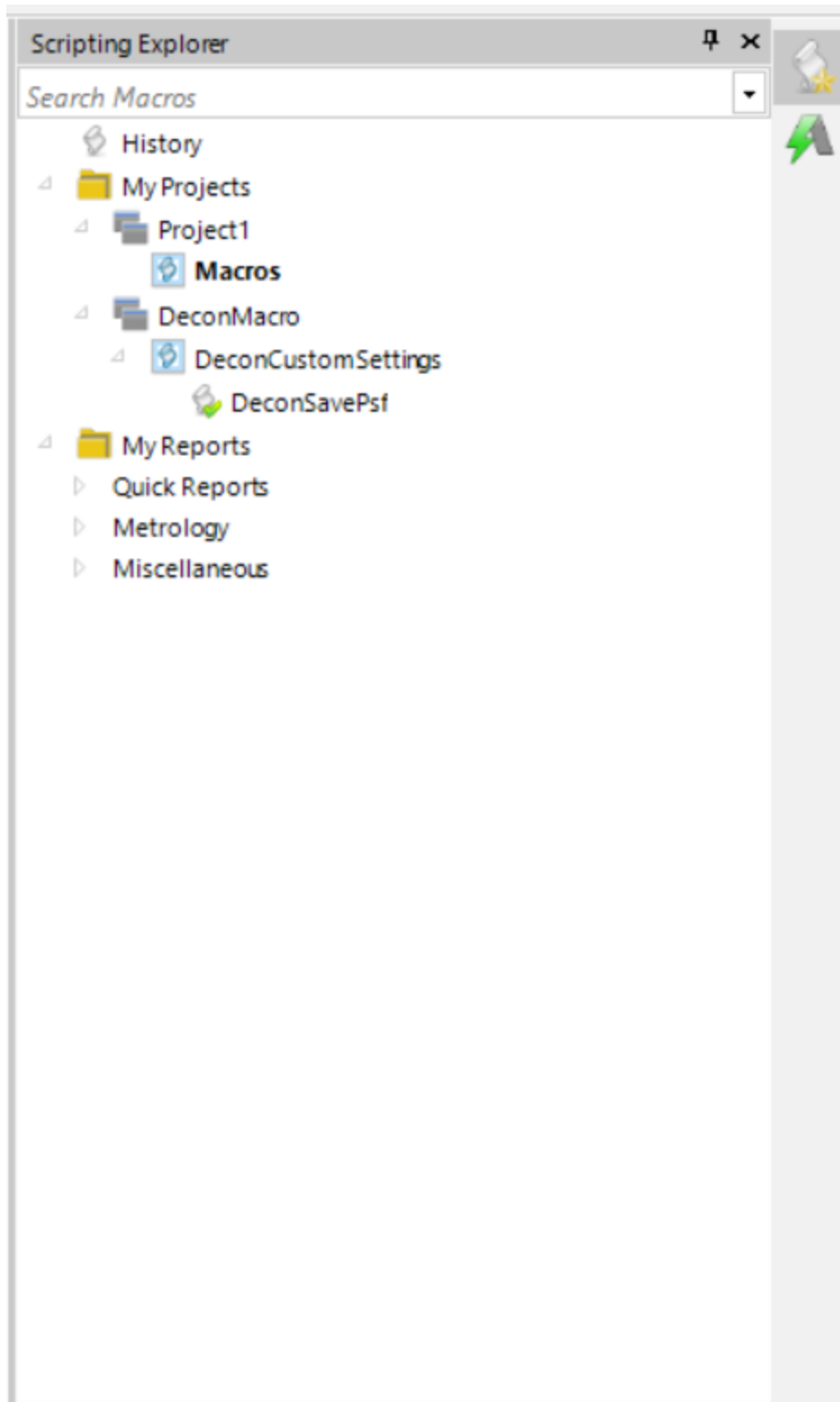


Figure 2. Scripting window within ImagePro. The window shows all open and available macros.

If running without the PSF printout, click deconvolve.

If running with the PSF printout, in the scripting explorer (above AutoQuant Deconvolution icon on the right side of side bar window) right click the DeconSavePSF (or macro option with the scroll; macros are denoted by the scroll icon) then click run.

- 7 Once the deconvolution is completed, the file will printout to ImagePro in a new tab with an asterisk by the file name and a number followed by an underscore preceding the file name ("10_" or "20_") to correspond with the iteration value.

Note

If using a macro to run the deconvolution, other files will printout the same way but with .psf following the file name that was printed out.

- 8 To gather intensity values, open the Intensity Tracking function from the Count/Size tab; on that tab, the Intensity function is the furthest on the right.

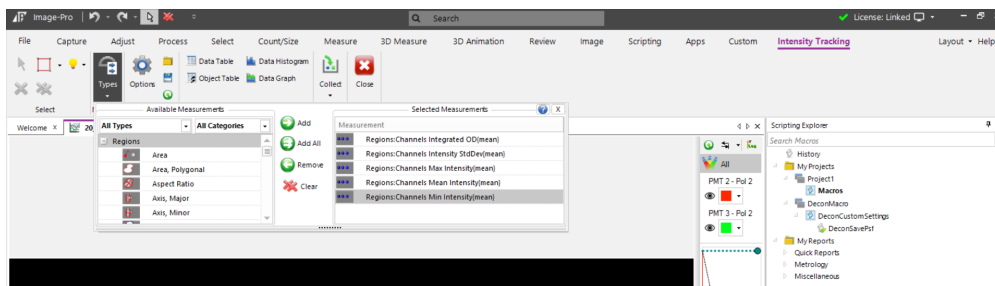


Figure 3. Intensity tracking ribbon showcasing the types of values that can be added or removed to the intensity tracking data collection.

- 8.1 Under types, as shown in Figure 3, select those for intensity mean, OD, maximum, minimum, and standard deviation of the channels.
- 8.2 In the select tab of the ribbon, use the square/rectangle option to select the entire image (or to select smaller regions of interest).
- 8.3 After the selection is made, click the collect button.
- 8.4 Either select Data Table in the data Views section of the ribbon, or look at the bottom of the image if it is already open (Figure 4).

- 8.5 Using the Excel function, a worksheet can be made, or the collected data within ImagePro and then can be copied to the computer's clipboard and then pasted into an existing data collection method.



Figure 4. The data table shown provides the location from which collected values can be exported using the Excel sheet function; or saved and exported as text file using the save function.

- 9 Repeat for all images and regions of interest.

Calculating SNR

- 10 Using the data collected from the steps described above, isolate the minimum and mean intensity values for each image and each channel.
- 11 The ImagePro intensity print out included sums for each column; these can be used for total or overall image SNR values. For subsets, select an n and isolate that n (51 for this experiment) in the values.

Note

The n should match a range from the image that best suits the following:
 Constraint i: overall stack must exceed 70 slices (z-planes)
 Constraint ii: ROI cannot be within 5 µm of the extremes of the stack (5 µm x image step size = # of planes to avoid at the top and bottom of the stack)
 Constraint iii: must contain a minimum of 1 full pollen grain within ROI of interest
 Constraint iv: size of pollen must be between 8-25 µm in diameter

- 12 To calculate the channel SNR, for the subset or overall image, sum the mean intensity values and divide that by the sum of the minimum intensity values for that channel.
- 13 To calculate the overall SNR for the subset or overall image, sum both channel mean intensity values and divide that by the sum of both channel minimum intensity values.

Note

An SNR over 5 is the goal.

Data Analysis: Prism

- 14 Open Prism and select Grouped under the Create column on the opening window.

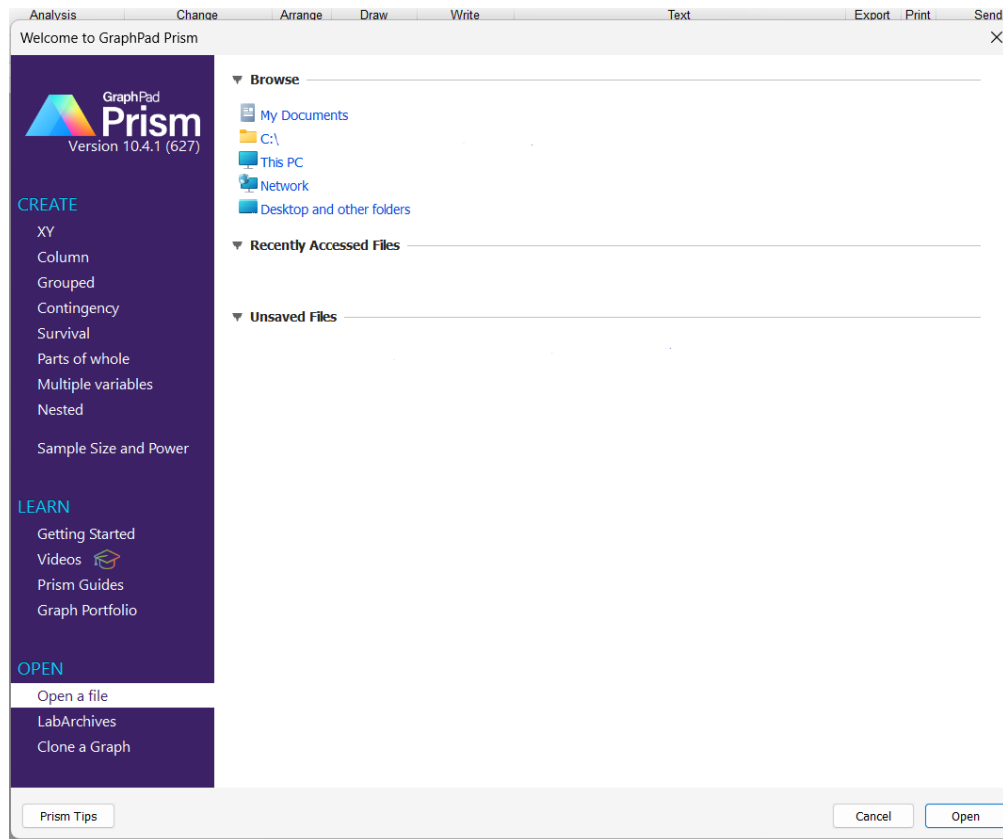


Figure 5. Opening selection window from Prism. This window provides initial selections for data type as well as easy access to previous files.

- 15 Insert data from the ImagePro Intensity collections with the values going across the page (across the rows) rather than down the page (within the column). An example is illustrated in Figure 6.

	A:1	A:2	A:3	A:4	A:5	A:6	A:7	A:8	A:9	A:10	A:11	A:12	A:13	A:14	A:15
1 RAW PMT2	314.217	313.891	314.451	314.391	312.416	316.200	315.611	315.669	315.335	317.486	317.849	316.794	318.399	316.287	319.905
2 RAW PMT3	273.133	273.490	273.374	274.190	274.035	274.558	274.273	274.327	275.474	275.004	274.651	276.668	275.787	275.485	275.331
3 Title															
4 Decon PMT2	192.990	192.990	193.503	193.503	194.103	194.103	194.103	194.103	194.661	194.661	194.827	194.827	195.913	195.913	194.527
5 Decon PMT3	307.807	307.807	308.087	308.087	309.229	309.229	310.008	310.008	307.261	307.261	308.809	308.809	308.204	307.644	308.324
6 Title															
7 AO RAW PMT2	312.740	315.075	314.916	313.982	317.487	314.891	316.385	318.311	316.025	316.899	316.749	319.977	317.688	317.283	320.269
8 AO RAW PMT3	273.971	273.366	274.785	273.580	273.952	274.086	272.635	274.790	273.368	275.099	273.663	273.664	274.457	274.058	275.926
9 Title															
10 AOD PMT2	208.048	208.048	208.382	208.382	207.205	207.205	206.914	206.914	208.728	208.728	207.093	207.093	207.803	207.803	209.661
11 AOD PMT3	319.472	317.866	316.865	318.933	320.359	319.492	318.032	318.189	319.703	319.582	319.001	318.159	318.645	319.893	320.458
12 Title															
13 Title															
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26 Title															
27 Title															

Figure 6. Data is in a grouped table with each cell containing one intensity value (for this experiment the mean of the image) grouped into media types (a, b, c) across the columns and grouped per imaging condition and channel down the rows.

16 To analyze this data, for this experiment, a two-way ANOVA was used.

Parameters: Two-Way ANOVA (or Mixed Model) ×

Model Repeated Measures Factor names Multiple Comparisons Options Residuals

Data arrangement

Table format: Grouped		Group A		Group B		Group C	
		Title		Title		Title	
		A:Y1	A:Y2	B:Y1	B:Y2	C:Y1	C:Y2
1	Title						
2	Title						
3	Title						
4	Title						

Matching by which factor(s)?
☒ Each column represents a different time point, so matched values are spread across a row.
☒ Each row represents a different time point, so matched values are stacked into a subcolumn

Include interaction term?
☐ No. Fit a main effects only model (column effect and row effect only).
☒ Yes. Fit a full model (column effect, row effect, and column/row interaction effect).

Assume sphericity (equal variability of differences)?
☒ No. Use the Geisser-Greenhouse correction. Recommended.
☐ Yes. No correction.

Based on your choices (on all tabs), Prism will perform:
 - RM two-way ANOVA with the Geisser-Greenhouse correction, matched values are both stacked and spread across a row.
 - Šídák's multiple comparisons test, with individual variances computed for each comparison.

Learn

Cancel

OK

Figure 7. Analysis window to input groupings and parameters to run a two-way ANOVA.

Note

This ANOVA requires the same n per condition. This experiment had a subset of $n = 51$ images or planes from a z-stack.

16.1 Fill out the parameters for the **model**.

The window shown in Figure 7 provides all the options and choices needed to run the ANOVA. Sphericity for this experiment was not assumed. The choices shown were the ones made for that tab.

Note

Repeated measures is explained in 16.2.
Factor names should match experimental groupings.

16.2 Fill out the parameters for **multiple comparison** options.

The repeated measures, factor names, and multiple comparisons tabs depend on the experiment. For this experiment multiple comparisons were manually selected to ensure the PMT channels (different pollen signals) would not cross in comparisons (cross labels), and the full model was fit.

16.3 Fill out the parameters for multiple comparisons tests.

The **options** tab provides corrections for comparisons (Sidak for this experiment), p value adjustments, and additional results that can be chosen.

16.4 Fill out the parameters for residuals.

The residuals tab offers extra graphical options to printout based on the residual values.

16.5 Run the analysis.

Note

Significance of 0.05 was set using Greenhouse-Geisser's epsilon test and Sidak's multiple comparison test.

17 After running the analysis, Prism prints out a result page showing the numerical results ,with additional tabs showing the Sidak's and Greenhouse-Geisser's results. Graphs can be created from here.

Protocol references

Simar, L. & Rosa-Molinar, E. (2025). "Benchmarking Assessment and Implementation of an Imaging Phantom in Nonlinear Optical Microscopy." Unpublished manuscript, *Current Protocols*.



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GraphPad: Prism