

Oct 01, 2025

Image Analysis for Starch Quantification in Histochemically Stained Flower Tissues using FIJI (Image J)

DOI

<https://dx.doi.org/10.17504/protocols.io.q26g7ndy9lwz/v1>

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Fruit trees



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Protocol Citation: Erica Fadón, Javier Rodrigo 2025. Image Analysis for Starch Quantification in Histochemically Stained Flower Tissues using FIJI (Image J). **protocols.io** <https://dx.doi.org/10.17504/protocols.io.q26g7ndy9lwz/v1>

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Protocol status: Working

We use this protocol and it's working

Created: September 26, 2025

Last Modified: October 01, 2025

Protocol Integer ID: 228231

Keywords: Reproductive plant biology, starch, flowers, microscopy, image analysis, FIJI, image analysis for starch quantification, starch in specific flower tissue, starch detection, starch quantification, histochemically stained flower tissue, indirect quantification of starch, specific flower tissue, source software program for scientific image processing, image analysis with proprietary software, scientific image processing, based image analysis, sample preparation for microscopy, gray scale calibration for optical density, image acquisition, main storage carbohydrate in plant, flower development, important role in flower development, gray scale calibration, specific tissue, optical density, main storage carbohydrate, imagej

Abstract

Starch is the main storage carbohydrate in plants and plays an important role in flower development, being accumulated in specific tissues prior to development. Starch can be easily visualized in histological sections stained with IKI; however, quantifying this starch in such small and specific tissues remains challenging. This issue has previously been addressed by indirect quantification of starch through computer-based image analysis with proprietary software.

In this protocol, we present a detailed step-by-step adaptation to perform an equivalent analysis using FIJI (ImageJ), an open-source software program for scientific image processing and analysis. The protocol is divided into three main stages:

- (i) sample preparation for microscopy: fixation, paraffin wax embedding, sectioning, staining, and mounting;
- (ii) image acquisition: microscopy and camera settings; and
- (iii) image analysis for starch quantification: selection of the measuring area, starch detection, black-and-white transformation, gray scale calibration for Optical Density, and measurement of the integrated density.

This protocol provides a reproducible workflow for the relative quantification of starch in specific flower tissues over time or under different experimental treatments.



Before start

Plant material preparation for microscopy

- Fix plant material in a fixative solution of ethanol/acetic acid (3:1) for at least 24 h at 4 °C. Then, discard the fixative and add 75% ethanol to be conserved at 4 °C until use.
- Dehydrate the samples in a tertiary butyl alcohol (TBA) series (85%, 95%, and 100%, v/v; and 3x with pure TBA) in a drying stove at 30 °C. Repeat it twice and incubate the last change for at least 4-6 days.
- Place each sample on a small metal base mold over a heat surface, embedded in paraffin wax, and place the embedding cassette. Place it over a cold surface and remove the block once the wax is solidified.
- Section each paraffin block at 10 µm in a rotatory microtome and place the sections onto the glass slide covered with Polylysine solution (1mg/ml in Tris) and dry them on a heat surface at 35 - 40 °C.
- Dewax and rehydrate the sections on the slides: histological clearing agent (3 times by 10 min), ethanol series (100%, 70%, 40%, v/v), and a final distilled-water wash (2 min each).
- Staining and mounting the sections of interest: Apply a drop of fresh I₂KI (2 g of potassium iodide (KI) and 0.2 g of iodine (I₂) in 100 mL of distilled water) over the section(s) for about 5 min and then discard the excess of stain by absorbing it with a blotting paper. Apply a small drop of a synthetic mounting media, place a small cover glass on top, and press hard.

Image acquisition: All images must be acquired under the same conditions, using consistent microscope and camera settings to ensure comparability across all experiments.

- 1 **Microscope settings** (the microscope I use is a Leica DM2500)
 - Light transmission bar: intermediate position (you can see through the eyepieces and the camera).
 - No filter
 - DIC: none.
 - Condenser: focus and aperture set at 10x
 - High light intensity: i.e. 8.
- 2 **Acquire all the images of the experiment** under the same conditions, using consistent microscope and camera settings to ensure comparability across all experiments.
- 3 **Acquire four control images** in the area without sample but with slide, medium, and coverslip (repeat this step every time you are going to acquire images):
 - A white image: without filters, representing 100% light transmission.
 - A light gray image: with a neutral-density filter ND2, which allows 50% of light transmission.
 - A dark gray image: with a neutral-density filter ND4, which allows 25% of light transmission.
 - A black image: without light.
- 4 **Camera settings:** Focus a section and then move to an area without sample (slide, medium, and coverslip) at the final magnification (in my case x40) and open the histogram and camera properties:
 - White balance: Camera properties → Image → White balance → Manual (leave on "manual" so these values remain fixed)
 - Exposure time: the exposure time should be the limit of overexposure, thus the minimum time for which all three RGB colors reach 255 in the histogram.
 - If needed adjust the white balance again.
 - Acquire the images in .tiff format.

Image analysis I: Selecting regions of interest and areas with starch.

- 5 **Charge all the images** to analyze in FIJI.
<https://imagej.nih.gov/ij/docs/guide/user-guide.pdf>
- 6 **Select the measurement area** (a square or a rectangle).

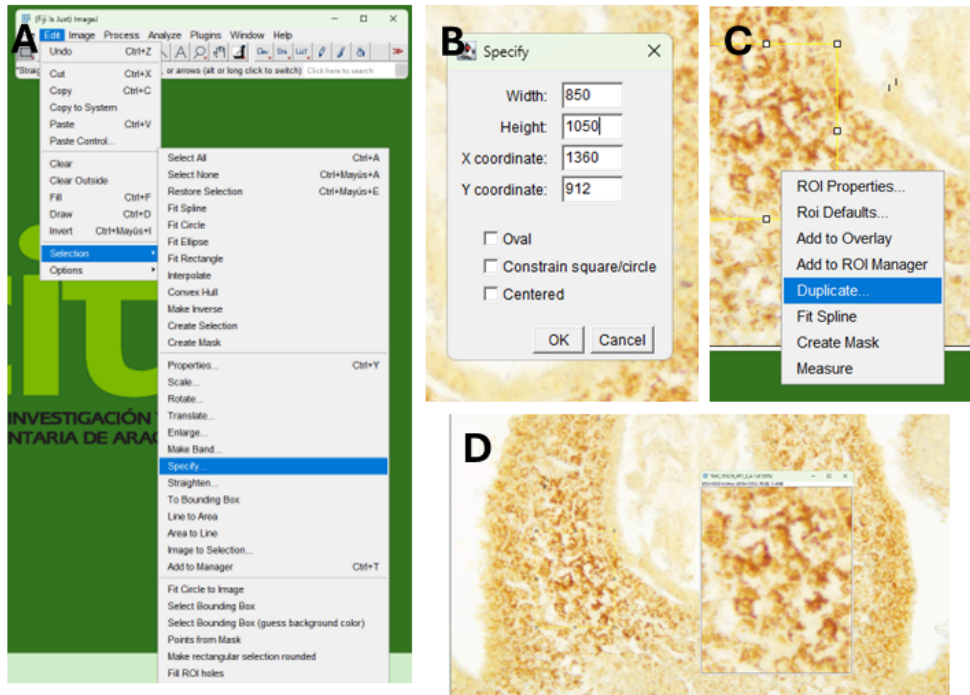
(A) Edit → Selection → Specify.

(B) Select the size of the area

(C) move the rectangle to the region to measure by clicking in the center and dragging → Right-click → Duplicate (a new image appears for further work).

(D) Save the new selected image in a new folder with the other selections.

Repeat C and D with the regions you want to analyze in the picture, and then do this with the other images.

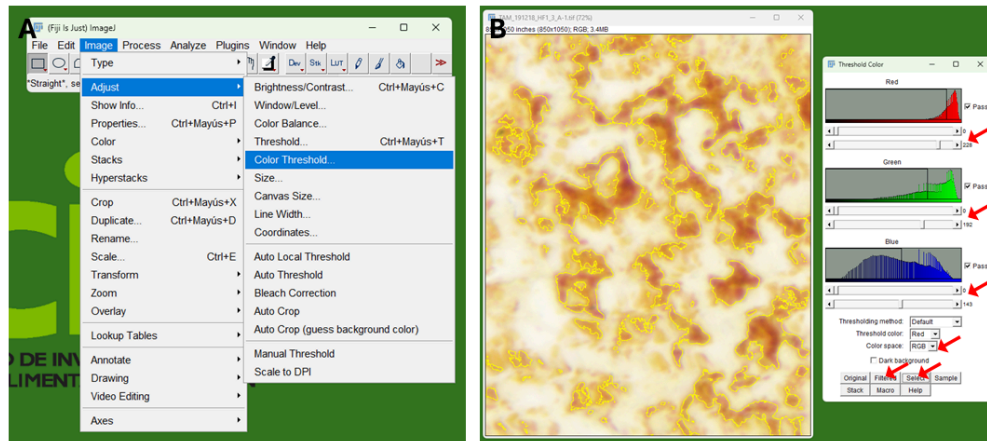


7 Select the areas with starch.

(A) Image → Adjust → Color threshold

(B) Color space → RGB. Then adjust the color bars till covering all the regions with starch (Filtered). And finally Select.

Save all these changes in a new folder (#starch_selection).



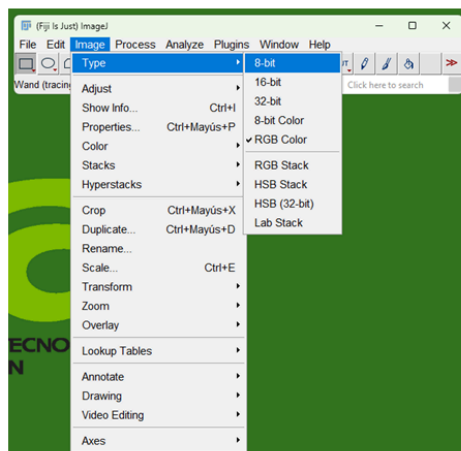
8 Convert image to black & white and save in a single folder (#B&W).

(A) You can do it with each image individually: Image → Type → 8 bit.

(B) Or convert all images at once: Process → Batch → Convert → Indicate the folders → Tiff 8-bit

- Convert the calibration images to black & white.

INDIVIDUALLY



ALL AT ONCE

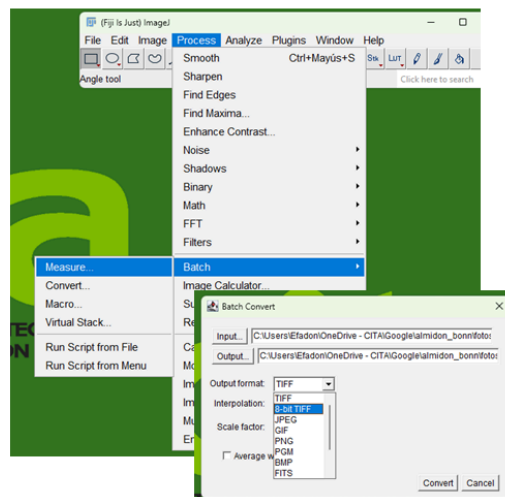


Image analysis II: measure the optical density of the starch areas with FIJI ImageJ

<https://imagej.nih.gov/ij/docs/guide/user-guide.pdf>



- 9 Load all images from the #B&W folder (calibration and analysis).
- 10 **Calibration: scale and grays**
<https://imagej.nih.gov/ij/docs/examples/calibration/>
- 11 Measure the open images (those calibrated for scale and color).
 - You can do it one by one: **Ctrl + M** or Analyze → Measure.
 - Or all at once (must be the folder where the images are open): Process → Batch (select the #B&W folder) → Measure.
- 12 The value of interest is **Integrated Density (IntDen)**, which takes the calibrations into account (in our case, only gray calibration since no scale is set).
Raw Integrated Density (RawIntDen) is the sum of pixel values without calibration. In this case, the pixel value is Mean, which, without calibration, is the gray value (0–255). When gray calibration is performed and the measurement is set to Optical Density (OD), the value of Mean is OD.
If the image is calibrated, **IntDen ≠ RawIntDen**.
To verify, once calibration is done, re-measure the control images (white and grays) and check that the Mean shows the calibration values set (0, 0.3, 0.6, 2.6).

Protocol references

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