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TITLE:

Staining the Cytoplasmic Ca^{2+} with Fluo-4/AM in Apple Pulp.

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SUMMARY:

Isolated protoplasts of apple pulp cells were loaded with a calcium fluorescent reagent to detect cytoplasmic Ca^{2+} concentration.

ABSTRACT:

Cytosolic Ca^{2+} plays a key role in plant development. Calcium imaging is the most versatile method to detect dynamic changes in Ca^{2+} in the cytoplasm. In this study, we obtained viable protoplasts of pulp cells by enzymatic hydrolysis. Isolated protoplasts were incubated with the small-molecule fluorescent reagent (Fluo-4/AM) for 30 min at 37 °C. The fluorescent probes successfully stained cytosolic Ca^{2+} but did not accumulate in vacuoles. La^{3+}, a Ca^{2+} channel blocker, decreased cytoplasmic fluorescence intensity. These results suggest that Fluo-4/AM can be used to detect changes in cytosolic Ca^{2+} in the fruit flesh. In summary, we present a method to effectively isolate protoplasts from flesh cells of the fruit and detect Ca^{2+} by loading a small-molecule calcium fluorescent reagent in the cytoplasm of pulp cells.

INTRODUCTION:

Ca^{2+} plays an important role in plant signal transduction and metabolism[1,2]. Further, it regulates fruit quality traits[3,4], including hardness, sugar content, and susceptibility to physiological disorders during storage[5,6]. Cytoplasmic Ca^{2+} plays an important role in signal transduction and regulates plant growth and development[7]. Disturbance of cellular calcium homeostasis can induce bitter pit in apples[8], brown spot disease in pears[9], and umbilical rot in tomatoes[10], affecting fruit quality and causing severe economic losses[3,11]. Calcium imaging has sufficient spatial and temporal resolution and is an important method for observing Ca^{2+} dynamics in living cells[12,13].

At present, there are two main methods for intracellular calcium imaging in live cells: one employs chemical small-molecular fluorescent probes[14], and the other is the gene encoding

sensor (GECI)[15,16]. Given the difficulty of establishing a stable transgenic system in fruit trees and longer fruit development, GECIs is unsuitable for fruit Ca^{2+} fluorescence imaging.

Small-molecule fluorescent probes such as Fluo-4/AM have a particular advantage: their AM ester form (cell-permeable acetoxymethyl ester derivative) can be readily bulk-loaded into living cells without the need for transfection, which makes it flexible, rapid, and non-cytotoxic[17]. Fluo-4/AM could successfully be loaded into the pollen tube of *Pyrus pyrifolia*[18] and Petunia,[19] as well as into guard cells[20] and root hair of *Arabidopsis*[21].

At present, there are few reports on the calcium fluorescence staining of pulp cells[22]. As an important mineral element, calcium plays a key role in the growth and quality control of tree fruits such as apples. Apple trees are globally recognized as an important economic species, and apples are considered a healthy food[23]. In this study, we obtained viable protoplasts from apple fruit pulp through enzymatic hydrolysis and then loaded small-molecule fluorescent reagents into the cytoplasm to detect Ca^{2+}.

PROTOCOL:

1. Protoplast extraction

1.1 Prepare the basic solution: 20 mM $CaCl_2$, 5 mM 2-(N-morpholino)ethanesulfonic acid, and 0.4 M D-sorbitol.

NOTE: The pH of the basic solution was adjusted to 5.8 with 0.1 M Tris buffer, filtered through 0.22 μm water-soluble filters, and stored at 4 °C.

1.2 Prepare the enzymatic solution: Mix 0.3%(w/v) Macerozyme R-10 and 0.5%(w/v) cellulase R-10 with the basic solution.

1.3 Add 0.5 mL of enzymatic solution into a 1.5 mL centrifuge tube. Pick a healthy and ripe apple. Then slice the pulp into 10 x 5 x 1 mm^3 size (**Figure 1A-1C)**.

1.4 Place the apple fruit pulp pieces into a 1.5 mL centrifuge tube containing enzymatic solution and then close the tube (**Figure 1D**).

1.5 Incubate the tube at 28 °C for 1 h, shaking at 70 rpm/min in a shaker in the dark.

1.6 Wash the pulp pieces. Aspirate all the enzymatic solution and then add 0.5 mL of the basic solution.

1.7 Centrifuge at 300 x *g* for 2 min at room temperature.

1.8 To obtain a protoplast suspension, aspirate the solution from the bottom of the centrifuge tube (**Figure 1E**).

2. Small-molecule calcium ion fluorescence staining

2.1 Prepare the Fluo-4/AM loading solution with 2 mM Fluo-4/AM, 20% F-127, and 10x phosphate-buffered saline (PBS: 80 mM Na_2HPO_4, 1.36 M NaCl, 20 mM KH_2PO_4, and 26 mM KCl) in a 1:1:2 ratio.

2.2 Add 1 µL of Fluo-4/AM loading solution to 99 µL of the protoplast suspension present in the 1.5 mL centrifuge tubes. Ensure that the final concentration of the fluorescent dye is 5 µM. Mix the solution and then close the tube.

2.3 La^{3+} treatment: Prepare 100 µM La^{3+} solution with 98 µL of the protoplast suspension, 1 µL of 1 mM La^{3+}, and 1 µL of Fluo-4/AM loading solution. Mix the solution and close the tube.

2.4 Incubate for 30 min at 37 °C in the dark.

2.5 Wash the protoplasts by centrifugation at 300 x *g* for 2 min at room temperature. Aspirate 70 µL of the solution and add 70 µL of the basic solution.

2.6 Incubate the protoplast suspension at 37 °C for 30 min to completely de-esterify.

2.7 Aspirate 15 µL of the protoplast suspension and drip onto a slide.

2.8 Observe under a fluorescence microscope (**Supplementary Figure S1**).

NOTE: Use a color camera with high sensitivity, i.e., 3.2 MP (2048 x 1536) CMOS sensor with 3.45 µm pixel resolution.

2.9 Select the GFP channel for imaging (20x). Set the brightness to 0.5.

NOTE: Illumination is adjustable-intensity LED light cubes with an integrated hard-coated filter set. The excitation wavelength of Fluo-4/AM is 490 nm.

3. Protoplast viability assay

3.1 Prepare the Fluorescein diacetate (FDA) stock solution: Dissolve FDA in acetone until the final concentration is 1 mg/mL.

3.2 Prepare the FDA working solution with 1 µL of the stock solution and 99 µL of acetone.

3.3 Add 1 µL of FDA working solution to 99 µL of protoplast suspension. Mix the solution by pipetting up and down and then close the tube.

NOTE: The final concentration of the FDA is 100 µg/L.

3.4 Stain at room temperature for 5 min in the dark.

3.5 Prepare the slides and observe under a fluorescence microscope.

3.6 Select the GFP channel for imaging (**Figure 1F**).

4. Image analysis

4.1 Analyze the acquired images using image analysis and spreadsheet software (e.g., Image-Pro Plus and Excel 2010).

4.2 Select two vertical diameters from the protoplasts to calculate fluorescence intensity (**Supplementary Figure S2**). Measure the fluorescence intensity of all protoplasts under different treatments was measured. For final processing, use photo editing software.

5. Statistical analysis

5.1 Perform statistical analysis using statistical software (**Supplementary Figure S3**). Data are presented in Mean ± SD. The student's *t*-test was used to analyze the differences between the experimental groups.

REPRESENTATIVE RESULTS:

Following the protocol described above, we used the enzymatic method to obtain viable protoplasts from the pulp (**Figure 1**). Some protoplasts had vacuoles, while others did not. While the protoplasts exhibited no fluorescence when the Ca^{2+} fluorescent indicator was not loaded into them. When Fluo-4/AM was loaded into the protoplasts, the cytoplasm, but not the vacuole, became fluorescent (**Figure 2**). This result indicated that Fluo-4/AM successfully stained Ca^{2+} in the cytoplasm and that no compartmentalization was observed[24]. Protoplasts were stained with FDA for 5 min and showed cytoplasmic fluorescence. This indicated that high temperature (37 °C) does not affect protoplast viability.

La^{3+}, a blocking agent of the Ca^{2+} channel[25], was added when Fluo-4/AM was loaded into protoplasts. At 100 µM, La^{3+} decreased calcium fluorescence intensity (**Figure 3**).

FIGURE AND TABLE LEGENDS:

Figure 1: Protoplasts obtained by enzymatic hydrolysis. (**A**) A piece was cut from a mature apple. (**B**) Protoplasts were extracted from pulp pieces. (**C**) The pulp was cut into 10 × 5 × 1 mm^3 pieces. (**D**) Pulp pieces were placed in centrifuge tubes containing 0.5 mL enzyme solution. (**E**) Protoplasts. Arrowheads point to the protoplast, and arrows indicate the vacuole in the protoplast. (**F**) Protoplasts were stained with FDA for 5 min at room temperature. (This figure has been modified from reference [22] [Horticulture research: Loading calcium fluorescent probes into protoplasts to detect calcium in the flesh tissue cells of *Malus domestica*].

Figure 2: Loading Fluo-4/AM and La^{3+} into the protoplasts. (**A**) Control: Intact protoplast without any loaded fluorescent probe. (**B**) Protoplasts loaded with Fluo-4/AM. (**C**) La^{3+} was added when the protoplasts were loaded with Fluo-4/AM. The final concentration of La^{3+} was 100 μM.

Figure 3: Statistical analysis of fluorescence intensity in the protoplasts. *** Indicate significant difference as per Student´s *t*-test (P <0.001). Vertical bars indicate ± SD. Each data point represents the mean of 20 protoplasts.

Supplementary Figure S1: Fluorescence microscope. (**A**) The overall appearance of the fluorescence microscope. (**B**) Settings page. Select 20x objective, GFP channel, and uniformly adjust the brightness to 0.5. (**C**) Fluorescence excitation region.

Supplementary Figure S2: Steps used to calculate the Ca^{2+} fluorescence intensity at protoplast of fruit cell. Fluorescence intensity units used in this study are based on previous reports[26-29]. The calculation process is as follows: (**A**) Open the protoplast fluorescence image in the software. Click on the **Measure** tool. From the dropdown menu select **Profile Line**. (**B**) Select **Circle** in the **Line Profile** window. Using this draw an ellipse at the protoplast. (**C**) In the **Line Profile** window now select **File | Export data** (**D**) Ensure that the blank spreadsheet is opened and import data by clicking **Data Export**. Use the 'average' function to calculate the average fluorescence intensity. Each treatment was repeated three times with more than 20 protoplasts each.

Supplementary Figure S3: Data statistics were performed using statistical analysis software. Paste the data into the table and click **Analyze** for data analysis.

Supplementary Figure S4: Extraction of protoplasts from the pulp of other varieties of apples. (**A**) Dounan. (**B**) Honey Crisp.

DISCUSSION:

In this study, viable protoplasts were obtained by enzymatic hydrolysis. Note that this method requires fresh apples. The present protocol allows for the rapid isolation of a large number of protoplasts from fruit pulp for use in research studies. The applicability of this method is not limited to 'Fuji'; the protoplasts of the apple pulp of 'Dounan' and 'Honey Crisp' can also be extracted through the same protocol (**Supplementary Figure S4**). The protoplast solution after enzymolysis contains cell debris, which was somewhat improved compared to previous methods. As an essential cell material, apple pulp protoplasts can be used for cell protein expression technology, single-cell sequencing, and other research.

There are many methods regarding chemical fluorescent reagent staining in plant cells[30]. For example, Fluo-3/AM was loaded at a low temperature (4 °C), such that it would enter the root tip cells[31]. Fluo-3/AM can also be loaded at a high temperature (37 °C) into the pollen tube[32]. Fluo-4/AM has successfully loaded into the pollen tube of *Pyrus pyrifolia* (25 °C for 15 min)[18] and the root hair of Arabidopsis (4 °C for 30 min)[33]. However, these methods are not suitable for staining apple pulp protoplasts. In this study, we successfully loaded fluorescent probes into

protoplasts at a high temperature (37 °C). In addition, the present method is simple, fast, accurate, and does not affect protoplast vitality. A critical step in the proposed method is to wash the stained protoplasts and incubate them for 30 min. After staining, the protoplasts should be washed. The washing will reduce the background fluorescence during observation so that the fluorescence intensity of the protoplasts can be counted more accurately. Incubate for 30 min to allow more probes to be loaded into the protoplasts. Note that it is essential to avoid light exposure during the loading.

La^{3+} is an important tool for studying Ca^{2+}. It binds to the Mg^{2+} catalytic site on the cytoplasmic membrane Ca^{2+}-ATPase, thereby inhibiting the steady-state turnover of Ca^{2+}-ATPase and blocking the Ca^{2+} transmembrane functioning[34]. La^{3+} treatment reduced cytoplasmic Ca^{2+}, and Fluo-4/AM could reflect this change, excluding the interference of other divalent cations and verifying the feasibility of chemical fluorescence reagents.

Although this loading method offers more advantages than other methods, it still needs to be further improved in transforming data results. Here, we estimated the relative cytoplasmic Ca^{2+} content by detecting the fluorescence value of protoplasts, which is qualitative data rather than quantitative. Therefore, it is particularly important to translate the staining results into specific cytoplasmic Ca^{2+} content in future studies, which could provide a research basis for analyzing fruit Ca^{2+} signaling.

In summary, the method described herein can detect cytoplasmic Ca^{2+} in apple pulp cells, thereby providing technical support for the related studies of fruit pulp-cell calcium.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare that they have no conflicts of interest with the contents of this article.

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Figure 1

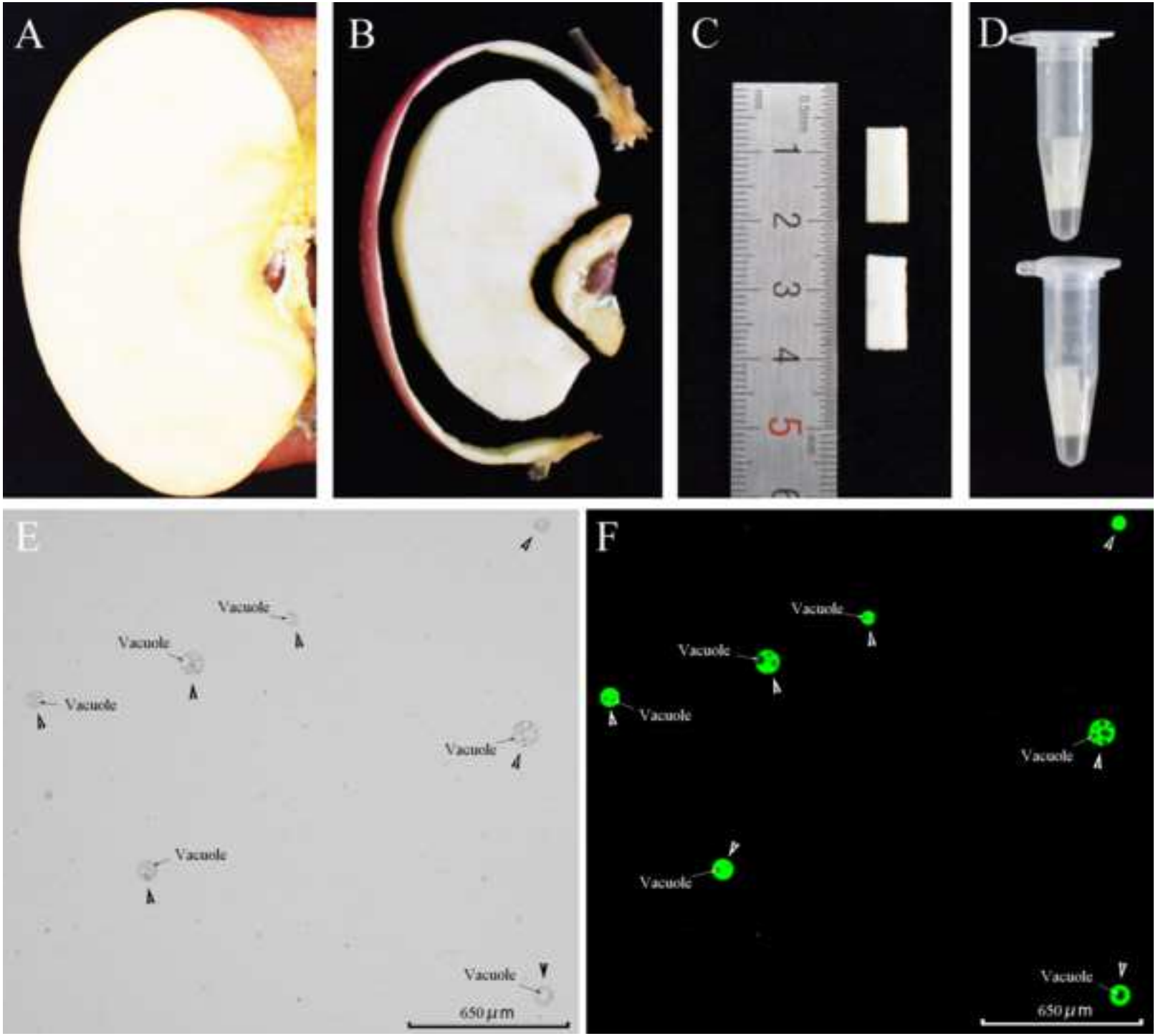
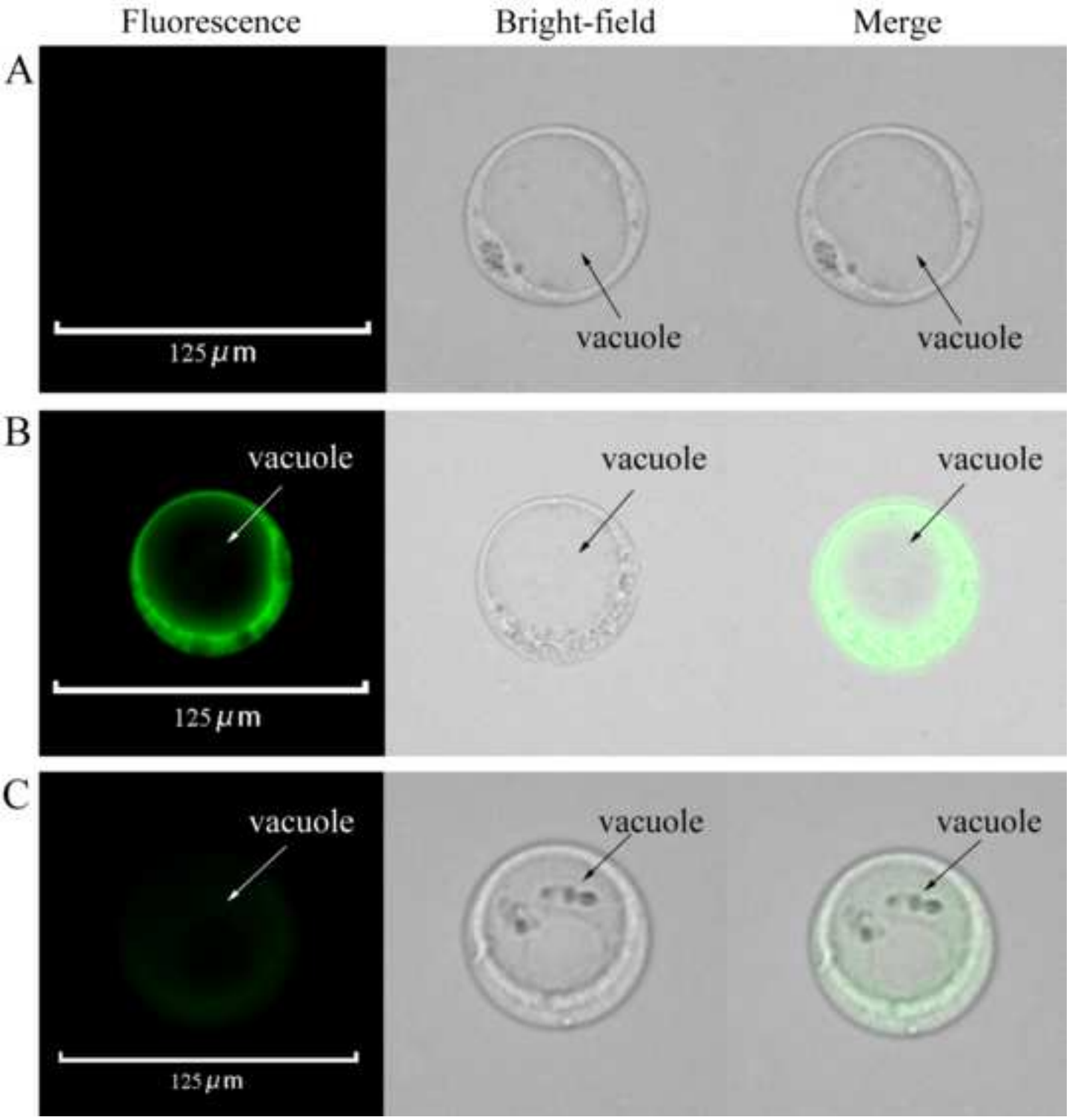


Figure 2



Figure

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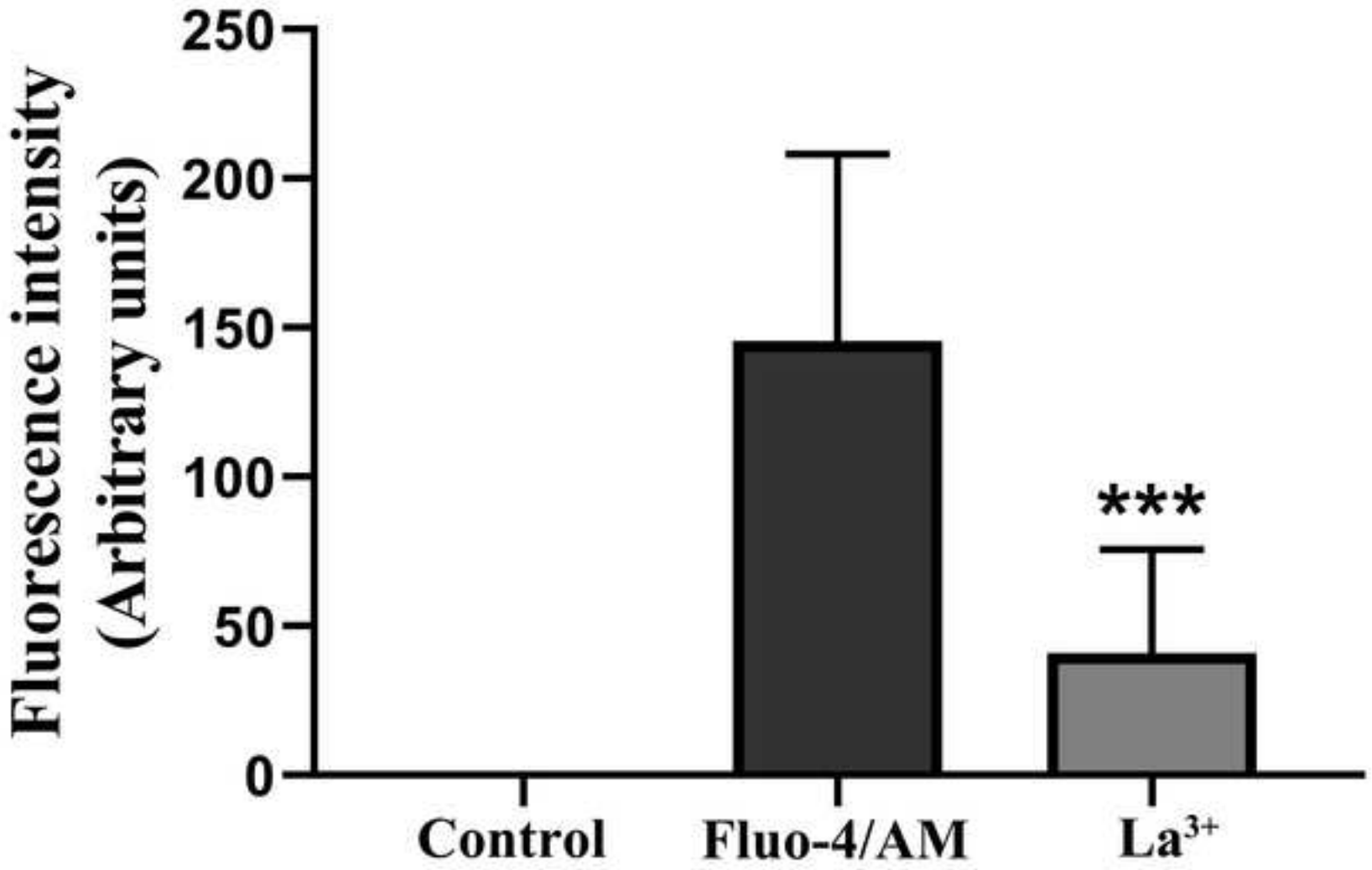


11/10/2021

Benjamin Werth, B.S.

Review Editor

JoVE Journal

Dear Editor:

We are very grateful to thank editors for their valuable comments on this paper and for giving us the opportunity to revise the paper, which is entitled “Method for staining the cytoplasmic Ca^{2+} of apple pulp with a small-molecule chemical fluorescent reagent. (JoVE62526R3)”. This will help improve the quality of the paper and provide some new research ideas for our future research. We valued the opportunity and checked the manuscript and video as a whole. Changes have been made as required. Modified parts of the manuscript have been highlighted in yellow. Once again, thank you very much for the editor and all reviewers.

Thank you for your consideration. I look forward to hearing from you.

Sincerely,

Lina Qiu
College of Horticulture, Qingdao agricultural university
qlina1996@163.com

Supplementary Figure S2: Steps used to calculate the Ca^{2+} fluorescence intensity at protoplast of fruit cell. Fluorescence intensity units used in this study are based on previous reports [26-29].

The calculation process is as follows:

(**A**) First step: Use the software Image-Pro Plus to open the protoplast fluorescence image, click on the "Measure" tool on the toolbar and then select "Profile Line" from the drop-down menu.

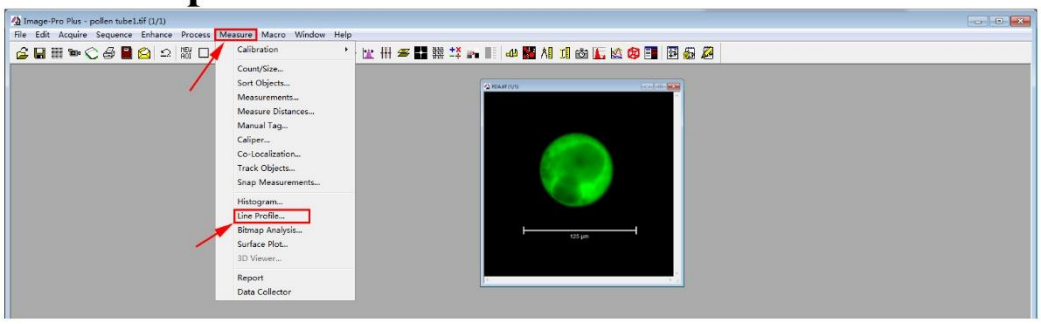
(**B**) Second step: Select the “Circle” in the “Line Profile” window and draw an ellipse at protoplast.

(**C**) Third step: Click the “File” in the “Line Profile” window and then click “Export data” from the drop-down menu.

(**D**) Fourth step: If the blank Excel form has already been opened, click "Data Export" to automatically import the data into the Excel form. Use the Excel function (AVERAGE) to calculate the average fluorescence intensity within the ellipse.

Each treatment was repeated three times with more than 20 protoplasts each.

A First step



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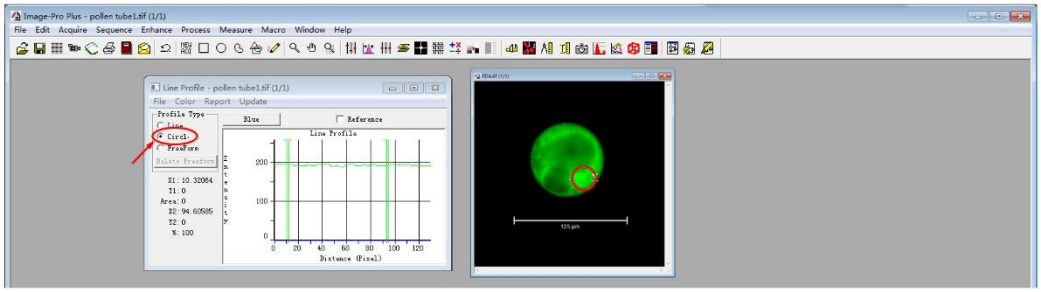

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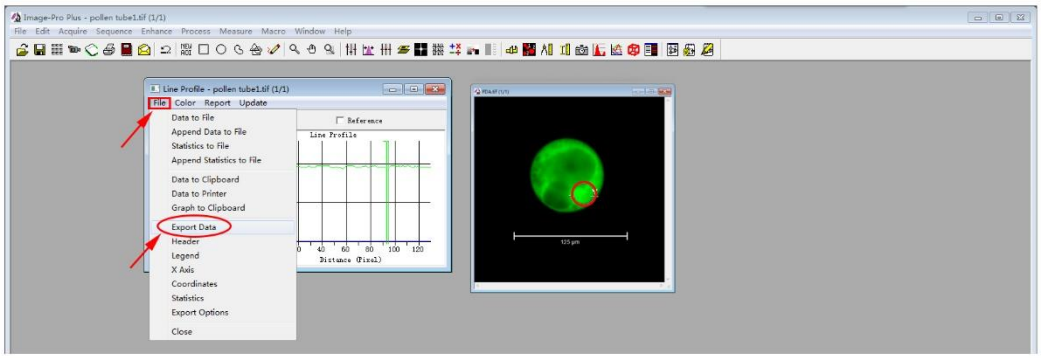

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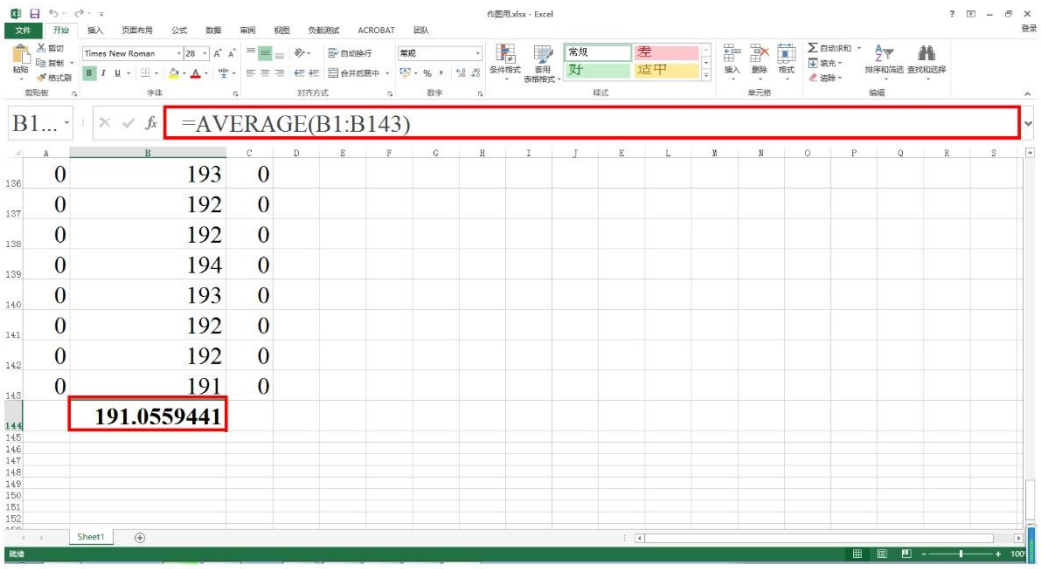

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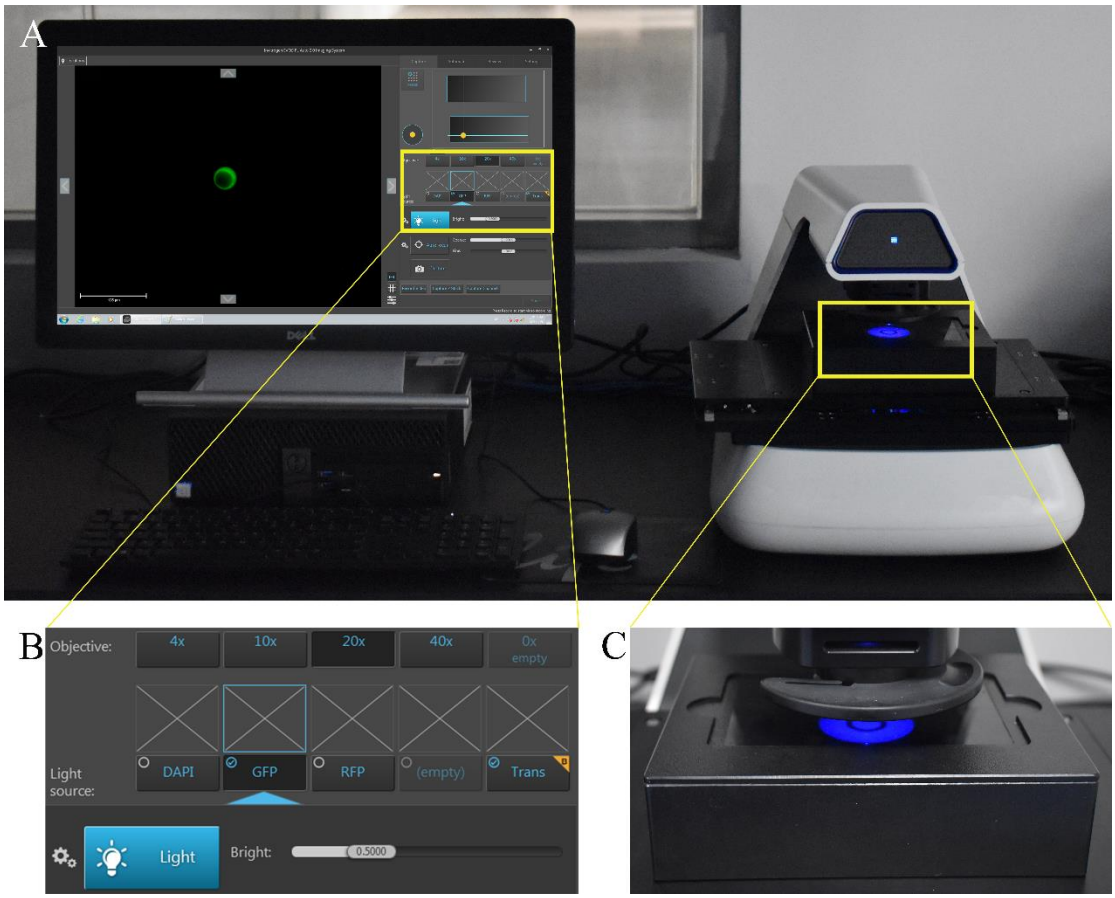
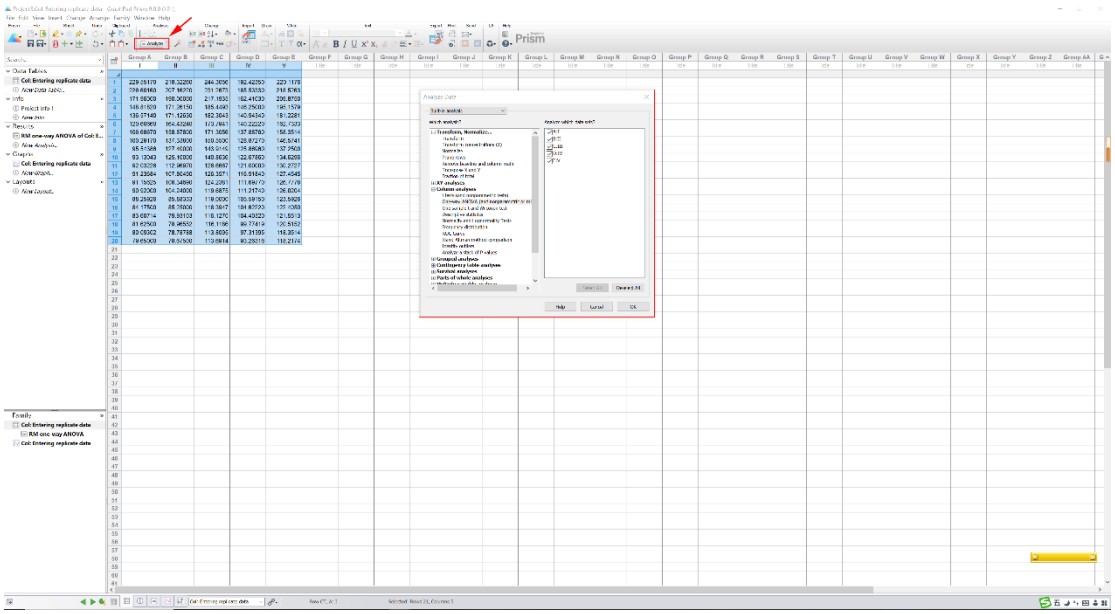
Supplementary Figure S1: Fluorescence microscope. (**A**) The overall appearance of the fluorescence microscope. (**B**) Settings page. Select 20x objective, GFP channel, and uniformly adjust the brightness to 0.5. (**C**) Fluorescence excitation region.



Supplementary Figure S3: Data statistics were performed using statistical analysis software (GraphPad Prism). Paste the data into the table and click "Analyze" for data analysis.



Supplementary Figure S4: Extraction of protoplasts from the pulp of other varieties of apples. A: ‘Dounan’. B: ‘Honey Crisp’.