STAR Protocols
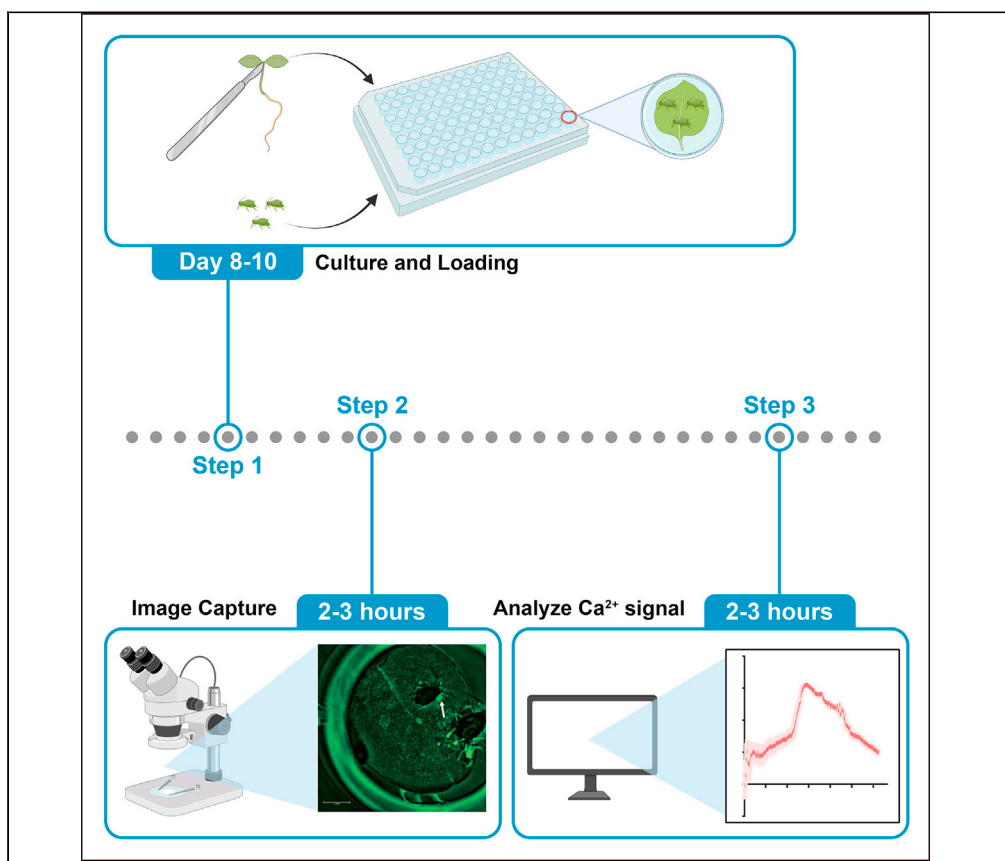
Protocol

Live imaging and quantitation of insect feeding-induced Ca^{2+} signal using *GCaMP3*-based system in *Nicotiana benthamiana*

Day 8-10 Culture and Loading

Step 1

Step 2

Step 3

Image Capture 2-3 hours

Analyze Ca^{2+} signal 2-3 hours

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Highlights

Highly efficient system for visualizing real-time Ca^{2+} flux in plants

Clearly display the calcium flow dispersive pattern after aphid's piercing-sucking

Step-by-step instructions to analyze Ca^{2+} flux dynamic by *GCaMP3* reporter system

Wounding evokes transient increases in cytosolic calcium (Ca^{2+}) concentration. Visualizing real-time Ca^{2+} flux provides new insights into Ca^{2+}-signaling pathways. Here, we outline a protocol to detect insect feeding-induced Ca^{2+} flux elevation in *Nicotiana benthamiana* leaves based on the *GCaMP3* reporter system by Leica fluorescence stereo microscopes (LFSM). LFSM combines super-fast manual screening with high-end imaging capabilities. Through this protocol, we can clearly observe the calcium flow after aphid's piercing-sucking. Additionally, we describe a protocol to quantify Ca^{2+} level using LFSM.

STAR Protocols

Protocol

Live imaging and quantitation of insect feeding-induced Ca²⁺ signal using *GCaMP3*-based system in *Nicotiana benthamiana*

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SUMMARY

**Wounding evokes transient increases in cytosolic calcium (Ca²⁺) concentration. Visualizing real-time Ca²⁺ flux provides new insights into Ca²⁺-signaling pathways. Here, we outline a protocol to detect insect feeding-induced Ca²⁺ flux elevation in *Nicotiana benthamiana* leaves based on the *GCaMP3* reporter system by Leica fluorescence stereo microscopes (LFSM). LFSM combines superfast manual screening with high-end imaging capabilities. Through this protocol, we can clearly observe the calcium flow after aphid's piercing-sucking. Additionally, we describe a protocol to quantify Ca²⁺ level using LFSM.
For complete details on the use and execution of this protocol, please refer to Wang et al. (2021).**

BEFORE YOU BEGIN

Plants have evolved stress-sensing machinery to initiate rapid adaptive stress responses. One of the main signals for plants in the perception of the environment is a transient rise in cytosolic calcium ion (Ca²⁺) concentration (DeFalco et al., 2017; Lee and Seo, 2021; Toyota et al., 2018). Ca²⁺ is a central player in the complex intracellular messenger system, and Ca²⁺ signaling mediates a wide range of biological processes (Lee and Seo, 2021). Although it has been found that mechanical wounding or insect feeding would lead to Ca²⁺ flux elevation and trigger multiple responses, it still lacks the clear image showing the calcium flow dispersive pattern at the moment of insect bites (DeFalco et al., 2017; Toyota et al., 2018; Vincent et al., 2017). To capture that moment, we use fluorescent calcium biosensor *GCaMP3* stable transgenic plants combined with Leica fluorescence stereo microscopes (M205 FCA, LFSM), which allows imaging at high speed with a higher signal-to-background ratio. Green fluorescent protein - calmodulin (GCaMP)-type calcium indicators have been used across multiple model organisms (Tian et al., 2009). Among them, GCaMP3 possesses greater protein stability, higher affinity for calcium compared to others. GCaMP3 is a circularly permuted EGFP molecule (cpEGFP), which is flanked at the N and C termini by Ca²⁺-dependent CaM-binding peptide M13 and calmodulin (CaM). In the absence of Ca²⁺, the protonated state of cpEGFP has low brightness. As Ca²⁺ levels rise, Ca²⁺-CaM complex is formed, which generates a deprotonated form of GCaMP by blocking solvent access to the chromophore, resulting in enhanced intrinsic fluorescence (Helassa et al., 2015). *GCaMP3* transgenic *N. benthamiana* plants have been used to monitor Ca²⁺ flux as described (DeFalco et al., 2017). This protocol aims to describe how to use green peach aphid (*Myzus persicae*) as a tool to study *N. benthamiana* calcium dynamics *in vivo* and in real-time by live imaging microscopes and how to quantify Ca²⁺ signal using an image analysis system (LFSM).

Preparation of *GCaMP3* transgenic plants

Ⓘ Timing: 8–10 days

The purpose of these steps is to prepare plant materials for analyzing Ca^{2+} signal response.

1. The ∼100 seeds of *GCaMP3* transgenic plants are surface-sterilized: seeds are rinsed in 1 mL 70% ethanol and shaken for ∼1 min in a 1.5 mL tube.
2. Rinse seeds in 1 mL sterile-deionized water for 4 times.
3. Sterilize the seeds with 1 mL sodium hypochlorite solution for 15 min.
4. Rinse seeds in 1 mL sterile-deionized water for 5 times.
5. Plant seeds evenly on a 100 mm × 15 mm square disposable petri dish with a 0.9–1 cm interval (about 49 seeds per dish) on MS nutrients containing 0.5% agar, 1% sucrose, pH 5.7–5.8.
6. Seal plates with micropore tape (3M).
7. Grow seedlings horizontally in a Safe PRX-450D incubator at 24°C–26°C with a 16 h/8 h light/dark photoperiod for 8–10 days.

⚠ CRITICAL: *GCaMP3* seedlings cultivated under abnormal growth conditions (too much high or low temperature, high humidity, pathogen pollution in the growth medium) were stressed and are not suitable for monitoring Ca^{2+} signal response. Therefore, seedlings must be cultivated under optimal growth conditions. In particular, excessive light in the growth chamber and excessive sucrose in growth medium should be avoided to prevent abiotic stress. The seedlings with two unfolded cotyledons (about 5 mm in diameter) are optimal for the experiment, neither too small nor too big leaves are suitable for the experiment. Besides, the seedlings for the experiment should display the normal developmental phenotype.

Reproducing aphids for *the* live imaging experiment

Ⓘ Timing: 6–8 days

The apterous adult aphids are selected to perform a Ca^{2+} signal live imaging experiment.

8. A green strain of *M. persicae* was maintained on the *Nicotiana tabacum* plant in a growth room at 26°C with a 16: 8 h light: dark photoperiod.
9. Apterous adult aphids from *N. tabacum* were placed on *N. benthamiana* plants to generate nymphs.
10. All aphids except nymphs were removed after 24 h.
11. The nymphs were allowed to develop into adults for 5–7 days on *N. benthamiana* plants.

⚠ CRITICAL: To collect enough apterous adult aphids for the following experiments, it is best to cultivate aphids on at least 10 *N. benthamiana* plants. For the aphid infestation assay, the aphids were gently transferred between plants by a soft lip brush. After infestation, the whole plant was covered with a cage of fine mesh gauze to avoid aphids moving to neighboring plants.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Murashige and Skoog (MS) Basal Medium	Duchefa Biochemie	Cat# DCF-M0222-0001
Agar	Solarbio	Cat# A8190

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sucrose	Solarbio	Cat# S8270
Sodium hypochlorite	Aladdin	Cat# S298682
Deposited data		
Table S1	Mendeley Data	https://doi.org/10.17632/cbgkh27fyk.1
Experimental models: Organisms/strains		
M. persicae apterous adult aphids (5–7 days)	Wang et al. (2021)	N/A
4-week-old *Nicotiana benthamiana*: WT	DeFalco et al. (2017)	N/A
8-day-old *Nicotiana benthamiana*: GCaMP3	DeFalco et al. (2017)	N/A
4-week-old *Nicotiana tabacum*	DeFalco et al. (2017)	N/A
Software and algorithms		
Leica Application Suite X (LAS X)	Leica Microsystems Inc	https://www.cellularimaging.nl/leica-las-x/
Excel	Microsoft	https://www.microsoft.com/en-us/microsoft-365/excel
Prism7	GraphPad	www.graphpad.com
Others		
Plates, petri dish 100mm✕15mm	Enzo	Cat# CSP-229656
96-Well plates	Corning	Cat# 3590
1.5 mL tubes	Axygen	Cat# MCT-150-C
Micropore tape (3M)	Easybio	Cat# BE6822
PRX-450D incubator	Safe	PRX-450D
Soft lip brush	N/A	N/A
Leica M205 FCA fluorescence stereo microscopes	Leica Microsystems	N/A
Leica DFC9000 GT digital camera	Leica Microsystems	N/A

MATERIALS AND EQUIPMENT

Sodium hypochlorite solution		
Reagent	Final concentration	Amount
Sodium hypochlorite (NaClO)	5% V/V	1 mL
ddH$_2$O	n/a	19 mL
Total	n/a	20 mL

Prepare the working solution on the day for sample processing.

| MS horizontal plates containing MS nutrients, 1% sucrose, pH 5.7–5.8. Store MS plates at 4°C for up to 1 week.		
Reagent	Final concentration	Amount
Murashige and Skoog (MS) Basal Medium	n/a	4.34 g
Agar	0.5%	5 g
Sucrose	1%	10 g
ddH$_2$O	n/a	Up to 1 L
Total	n/a	1 L

Adjust the MS nutrients pH to 5.7–5.8 with 0.5 M KOH, and autoclave at 110°C for at least 30 min. Cool to ~60°C and pour into a disposable Petri dish after autoclave.

STEP-BY-STEP METHOD DETAILS
Preparing living leaves and aphids for imaging

⏲ Timing: 26–28 h

The purpose of these steps is to prepare living leaves and aphids for analyzing Ca^{2+} signal response.
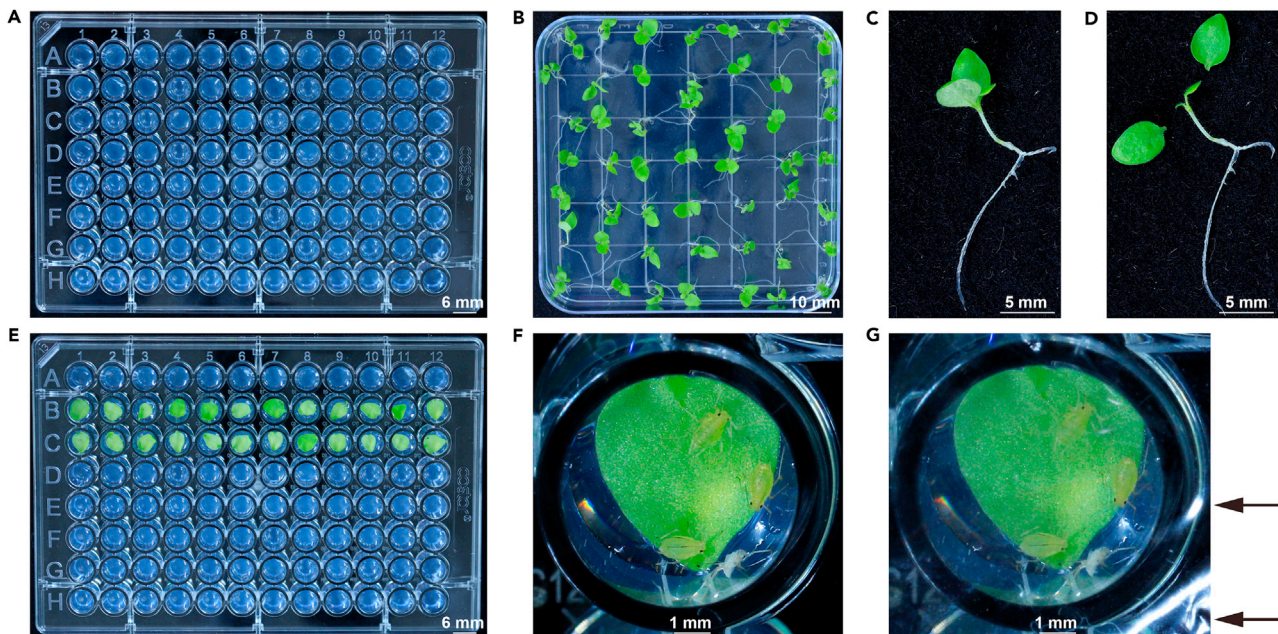
Figure 1. Preparing living leaves and aphids for imaging

(A) Fill each well of sterile polystyrene 96 well-flat bottom tissue culture plate with 0.2 mL solid MS nutrients.

(B–D) Both cotyledons were cut off from 8-day-old *GCaMP3* seedlings.

(E) The removed cotyledons were then carefully placed horizontally in each well of the 96 well-plate by sterile forceps.

(F and G) Three starved apterous adult aphids from the plastic petri dish were gently transferred to the cotyledon surface in each well by soft lip brush and rapidly wrapped the 96-well plate in plastic wrap to prevent the aphids from escaping. Arrows indicate that the 96-well plate was wrapped with plastic wrap.

1. Fill each well of sterile polystyrene 96 well-flat bottom tissue culture plates with 0.2 mL solid MS nutrients (Figure 1A).
2. Both cotyledons were cut off from 8-day-old *GCaMP3* seedlings by a sterile scalpel blade (Figures 1B–1D).
3. The removed cotyledons were then carefully placed horizontally in each well of the 96 well-plate by sterile forceps (Figure 1E).
4. Cover the 96-well plate with sterile plastic wrap.

    Note: Operations are performed in aseptic condition with ultra-clean benches.

5. Place the plates in the PRX-450D incubator at 24°C–26°C with a 16 h/8 h light/dark photoperiod for 24 h.

    △ CRITICAL: Physical damage, including cutting, will transiently trigger the calcium flow in leaves and affect the follow-up observation. To eliminate this effect, the removed cotyledons should be maintained on MS medium in the incubator for at least 24h.

6. After 24 h, take out the plates from the incubator and uncover the plastic wrap for 1 h to ventilate the plate and remove the water vapor from the cotyledon surface.
7. Apterous adult aphids from *N. benthamiana* were gently transferred into an empty plastic petri dish by the soft lip brush to get starvation treatment to increase the aphid's sap-sucking frequency.

    △ CRITICAL: To increase the aphid's sap-sucking frequency, we often starved the aphids for 4 hours before observation.
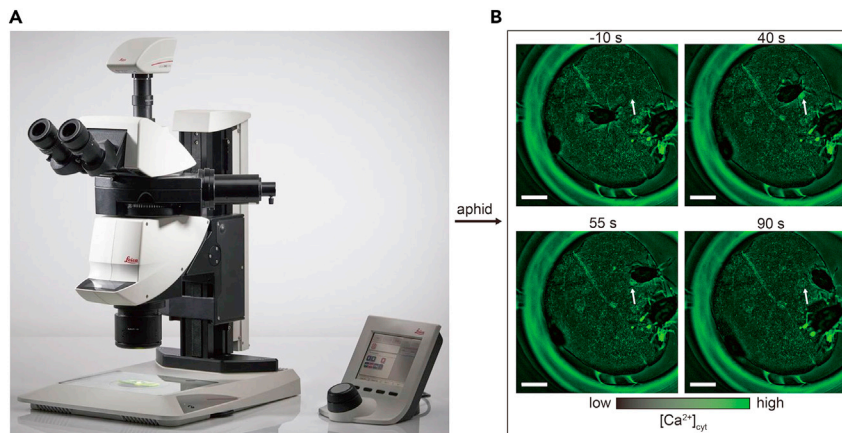
Figure 2. Detecting calcium signals using LFSM
(A) Leica M205 FCA fluorescence stereo microscopes (LFSM)
(B) Shoot the paragraphs to record the fluorescence dynamics at the area of aphid's bites. Scale bar = 1 mm.

8. Three starved apterous adult aphids were gently transferred to the cotyledon surface in each well (except four wells as the control group) by soft lip brush and rapidly wrapped the 96-well plate in plastic wrap to prevent the aphids from escaping (Figures 1F and 1G).

Detecting calcium signals using LFSM

© Timing: 2–3 h

In this section, the settings and parameters of microscopy for detecting GFP fluorescent, which indicates Ca^{2+} signal dynamic in living cotyledons, are outlined in Table S1.

9. For imaging experiments, we use a Leica M205 FCA fluorescence stereo microscopes (LFSM) equipped with a pair of eyepieces 10×/23B, ET GFP filter, and "thunder" image processing system (Figure 2A).
10. Open Leica Application Suite X (LAS X) software and set the microscope parameters as follows (Methods video S1):
    a. Acquisition mode was xyt.
    b. Set the filter as ET GFP.
    c. Images were acquired at 16 bit and with 1024 × 1024 pixels (Image Format: 2 × 2 Binning).
    d. Exposure time is 300 ms, and the gain is 2 (Gain List: low nose & high well).
11. Select "TL light" or "SLI light" as optical mode (choose "SLI light" mode if the microscope stage was covered by dark black paper as shown in Methods video S1) to operate bright-field illumination. To avoid overexposure, the intensity of TL light or SLI light should be set to a minimum degree and then gradually increased. The illumination settings of the example dataset were acquired as follows:
    a. TL contrast mode is TL-DF with 53 positions.
    b. TL light intensity is 1000.
12. Place the 96-well plate with cotyledons and aphids on the microscope stage. Adjust the zoom lens to focus the view on the samples in one well under bright-field illumination (the zoom is about 2.0–3.0) and ensure the clear observation of the leaf in the control group (Methods video S2).
13. Detect the GFP fluorescence of leaves in the control group, and GFP fluorescence represents the background fluorescence intensity of *GCaMP3* leaves.

14. Once the parameters were set appropriately, gently move the 96-well plate to focus on the samples with aphids. Set the "Exposure time" as 300 ms and define the recording time (for example, 5 min or until the calcium flow vanishes). Capture the emitted fluorescence using a Leica DFC9000 GT digital camera (sCMOS) (Methods video S3). Shoot the paragraphs to record the fluorescence dynamics at the area of aphid's bites (Figure 2B).

   Note: Control group was used to adjust LFSM parameters for eliminating the background "noise".

   ⚠ CRITICAL: Since the aphids need time to adapt to the new environment, it should be patient to wait for enough time before shooting. To precisely capture the moment of aphid's bites, it's better to observe the aphids' moment and their antenna. Aphid always keeps moving except for sucking, and they would sway their antenna backward to bodies when they are biting the leaves. Therefore, these behavior signatures could indicate the following "bites".

Exporting video and analyzing Ca^{2+} signaling using LFSM software

⏲ Timing: 2–3 h

The purpose of these steps is to export video and quantify Ca^{2+} signaling using LFSM software.

15. The steps for generating and exporting a video by LFSM software are outlined below (Methods video S4):
    a. Open the LAX software (3.3.0.16799), choose the "Standard Configuration" pattern, and click the "OK" button (Figure 3A).
    b. In the next window, select "Open Project" in settings and load files of interest with the suffix.lif (Figures 3B and 3C).
    c. Click on "Process" – "Show Gallery" (status bar on the right) on the graphical user interface to view the captured images (Figures 3D and 3E).
    d. Click on "Annotations" to add directive symbols on pictures (Figure 3E).
    e. Select the start image of the video, click on "Set Start" in the middle menu (Figure 3F).
    f. In the same way, select the last image and click on "Set End" (Figure 3G).

   Note: To view an image clearly from the gallery, you can double click on the selected image to enlarge it (Figure 3E).

    g. Applying the settings, a new file containing the frames from "start image" to "end image" was generated.
    h. Right-click on the new file and select "Export" - "As MPEG-4" - "Frames/sec" to export the video (Figures 3H–3I and Methods video S5).

   Note: The larger number filled in "Frames/sec" would compress the file into a faster video.

16. The steps for analyzing Ca^{2+} signaling by LFSM software are outlined below (Methods video S6):
    a. Open the LAX software and load in the newly generated video (Figure 4A).
    b. Double click the video in the left status bar and select the "Quantify" module. Then click the "Stack profile" button in the pulldown menu of "Tools" and tick the box "All in one". Therefore, any selected region would be marked in every frame at the same position, and the fluorescence value of the selected area in every frame would be analyzed respectively (Figure 4B).
    c. Click the Rectangle or Oval icon in the upper right toolbar to choose the shape of the selected region. Then, drag the mouse button to select an area, and the fluorescence value
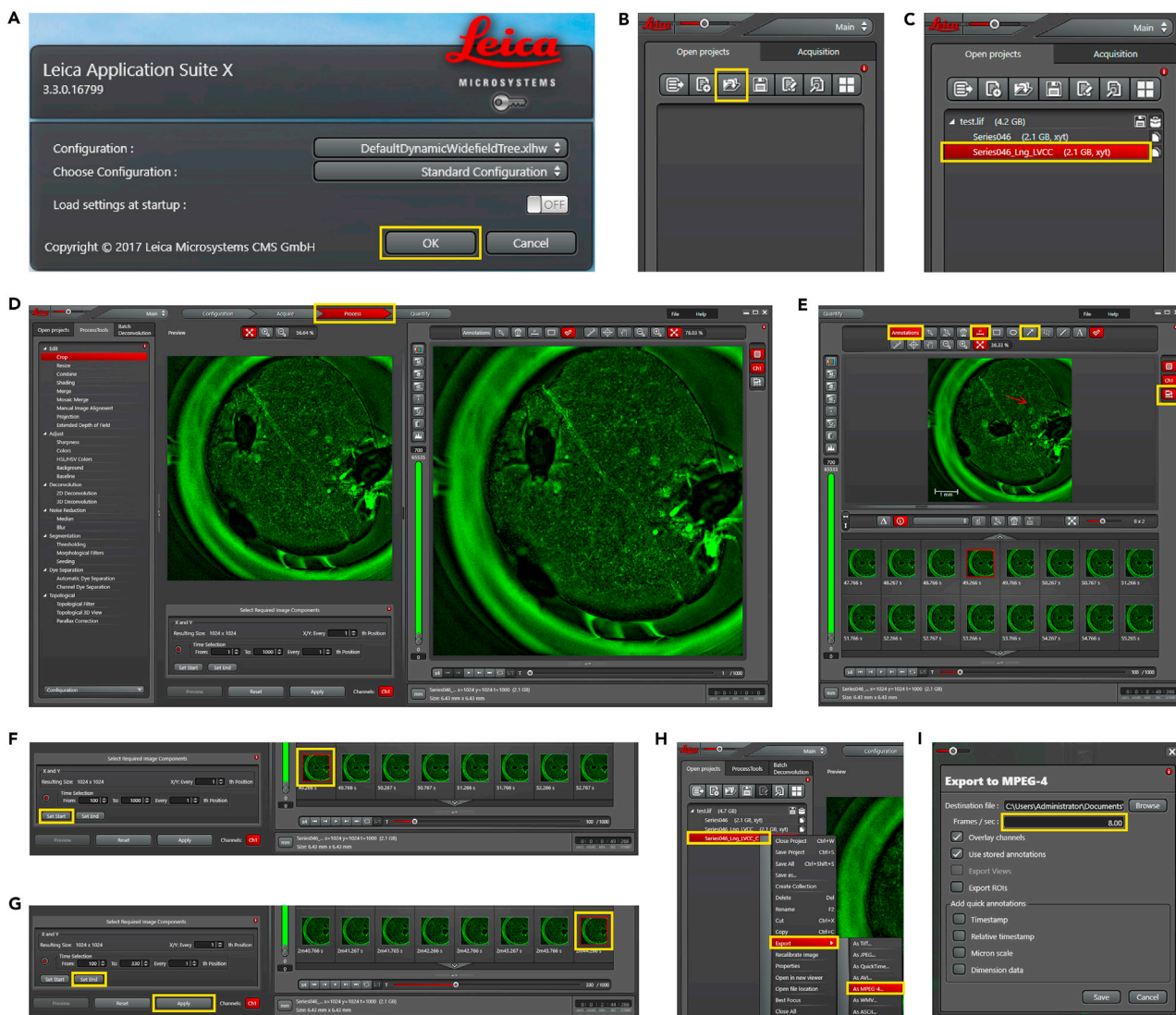
Figure 3. Overview of exporting videos using LFSM software

(A–C) Open the LAX software and load files of interest with the suffix.lif.

(D–G) Click on "Process" – "Show Gallery" to view the captured images. Select the start image of the video, click on "Set Start" in the middle menu. In the same way, select the last image and click on "Set End". A new file containing the frames from "start image" to "end image" was generated.

(H–I) Right-click on the new file and select "Export" - "As MPEG-4" - "Frames/sec" to export the video.

will be displayed in the selected region. Meanwhile, in the middle area of the interface, the mean intensity of fluorescence value of the selected region in every frame will be presented as a numerical point, and the points will be automatically linked as a curve with one color (Figure 4C).

d. The repetitive operations should be done to select multiple regions (closed to Ca^{2+} flux occurring position). There will be multiple curves with different colors in the middle area of the interface. Right-click on the curves, select "Export" and "Excel", and export all fluorescence values with its corresponding time data of the selected region in every frame as an excel file (Figure 4D).

e. To calculate the fractional fluorescence changes ($\Delta F/F$), the equation $\Delta F/F = (F - F_0)/F_0$ was used, where F_0 denotes the average baseline fluorescence determined by the average of F over the first 5 frames of the recording before aphids feeding. "F" means the fluorescence
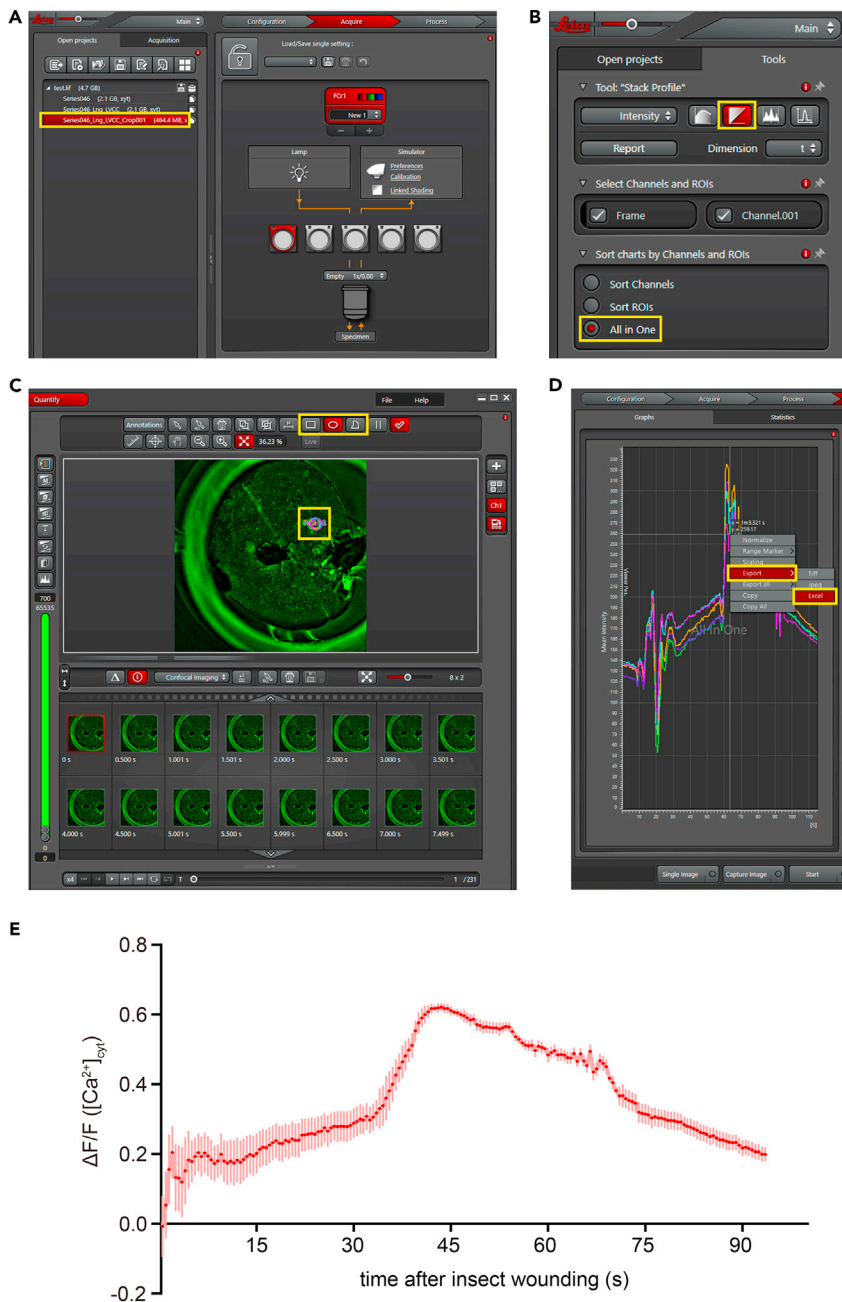
Figure 4. Overview of analyzing Ca²⁺ signaling by LFSM software

(A) Open the LAX software and load in the newly generated video.

(B) Click the "Stack profile" button in the pulldown menu of "Tools" and tick the box "All in one". Therefore, any selected region would be marked in every frame at the same position, and the fluorescence value of the selected region in every frame would be analyzed, respectively.

(C) Choose the shape of the selected region and select the aphid biting area.

(D) Right-click on the curves and select "Export", "Excel" to export all fluorescence values and its corresponding time data of the selected region in every frame as an excel file.

(E) The $[Ca^{2+}]_{cyt}$ signature variation in leaf during aphid feeding. Data are mean ± SD.

value. Every single selected region owns a ΔF/F ratio. Average the ratios of multiple regions and collect the data.

   f. Those data would be analyzed and made into Ca^{2+} flux dynamic curve mode by Prism7 software (Figure 4E).

EXPECTED OUTCOMES

Stress and stimuli such as mechanical wounding or insect feeding could provoke the transient dramatic Ca^{2+} flux elevation in plants (DeFalco et al., 2017; Lee and Seo, 2021; Toyota et al., 2018). Ca^{2+} signal transduction is known to be involved in a wide range of plant physiology, such as cellular response to biotic or abiotic stress, tip growth of roots and pollen tubes, the transition from vegetative to reproductive growth and organ development (Lee and Seo, 2021). Visualizing real-time Ca^{2+} levels provides important new insights into Ca^{2+}-signaling pathways. Through this protocol, the Ca^{2+} flux dynamic after aphid's piercing-sucking could be visualized lively and quantified precisely in *N. benthamiana* leaves, as shown in Methods video S5. This system is suitable for identifying insect-feeding-induced calcium flux, and applied for calcium flux detection of plant response to multiple stresses such as temperature variation, pathogen invasion, and pest infestation. Besides, this system may be used for reverse genetic screens to help identify receptors, sensors, or upstream regulators in Ca^{2+} signaling pathways by isolating the mutant plants which impair the Ca^{2+} flux elevation.

LIMITATIONS

Although *GCaMP3*-based Ca^{2+} fluorescence system provides a viable platform to lively image the Ca^{2+} flux dynamic after aphid's piercing-sucking in *N. benthamiana*, it still has the following limitations.

Due to the limited size of vision, this protocol is more suitable for lively imaging the transient Ca^{2+} flux dynamic on sub-region of cotyledons, but not for the whole plants. Therefore, it still needs to improve the protocol for imaging other organs, such as flowers, roots, or entire plants. Besides, the apterous adult aphids are tiny and unable to fly, suitable for the experiment, but it is still challenging to image Ca^{2+} signals with enormous insects.

Stimuli even slight effect could provoke the transient dramatic Ca^{2+} flux elevation, meanwhile, under lasers irradiation, aphids would reduce the feeding frequency due to the photophobia habit. Hence, it is a labor-intensive and time-consuming experiment that requires skilled researchers, expensive equipment, and enough space. Detailed experiments should be well designed in advance.

TROUBLESHOOTING

Problem 1
Strong Ca^{2+} signal "background noise" (steps 9–14).

Potential solution
The strong Ca^{2+} signal background could be caused: 1. Stimuli could provoke the transient dramatic Ca^{2+} flux elevation. 2. The microscopy is not on optimal work conditions. To avoid those situations, the following critical steps should be performed: 1. Seedlings must be cultivated under optimal growth conditions. In particular, excessive light in the growth chamber and excessive sucrose in growth media should be avoided to prevent abiotic stress. 2. Removed cotyledons should be maintained on MS medium in the incubator for at least 24 h before live imaging. 3. Apterous adult aphids from the plastic petri dish were gently transferred to the cotyledon surface in each well by a soft lip brush and rapidly wrapped the 96-well plate in plastic wrap. 4. The exposure time should be set to a minimum degree and then gradually increased. 5. The "thunder" molecule in LAX software is designed for processing images, applying that function could reduce the background fluorescence automatically by computational analysis.

Problem 2
The strong GFP fluorescence emitted from the plastic plate (steps 9–14)

Potential solution
It is worth noting that the plastic plate would generate strong GFP fluorescence. It is necessary to reduce the background strong GFP fluorescence if the Ca^{2+} flux-induced GFP fluorescence is not intense enough to observe. On the one hand, the dull black material such as a sheet of black paper could be spread on the microscope stage to cut down the reflected light. On the other hand, black microplates with clear bottom could be used to eliminate the plastic emitted fluorescence.

Problem 3
The vision is obscure and unable to capture the fluorescence signal precisely (steps 12–14).

Potential solution
To keep vision clear and precisely focus on the area of aphid's bites, we should focus the camera on the leaf before shooting. It had better keep only one well in sight to avoid the focal length difference between different leaves. Focusing should be operated under bright-field illumination and operation can be done with coarse adjustment followed by fine adjustment until the epidermis hair of leaf could be clearly observed in the vision. Then shut down the bright-field illumination and turn on the GFP fluorescence lamp. It should be noted that vision-shift from one well to another should be along with refocus.

Problem 4
Hard to capture the aphid feeding moment (step 14).

Potential solution
Since the aphids need time to adapt to the new environment, it should be patient to wait for enough time before shooting. Besides, under lasers irradiation, aphids would reduce the feeding frequency due to the photophobia habit. Therefore, it is better to starve the aphids for 4 h before observation. The experiment could be performed in the evening since we found aphids are more active in the evening or dark environment. Besides, we also found that aphid always keeps moving except for sucking, and they would sway their antenna backward to bodies when they are biting the leaves. Therefore, these behavior signatures could indicate the following "bites".

Problem 5
Ca^{2+} flux dynamic curve displays sharp vibration (step 16).

Potential solution
Once the sub-region of leaves is selected in one image, the mean intensity of fluorescence value of the selected region in every frame will be presented as a numerical point, and the points will be automatically displayed and exported. However, before these fluorescence values of its corresponding time are used to make the Ca^{2+} flux dynamic curve, an important factor should be considered and excluded: aphids like to move back and forth after biting, and their body could cover the fluorescence for some seconds. Therefore, the fluorescence value of the selected region in such frames should be abandoned to prevent instability.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yule Liu (yuleliu@mail.tsinghua.edu.cn).

Materials availability

All unique/stable reagents used in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

This study did not generate/analyze datasets. Table S1 data have been deposited to Mendeley Data: https://doi.org/10.17632/cbgkh27fyk.1.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.101040.

AUTHOR CONTRIBUTIONS

Y.W. and Q.G. wrote the manuscript; Y.L. reviewed and edited the manuscript; Y.W., Q.G., and L.H. prepared figures, tables, and videos; Y.W., Q.G., and F.H. prepared the graphical abstract.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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