Deciphering cell cycle phases of polyphenic tissues is an important challenge in understanding the cellular mechanism of polymorphism. We use flow cytometry to analyze cell cycle phases of short wings and long wings of the brown planthopper. This provides information on the arresting cell cycle phases in different wing forms. The protocol could be applied to analysis of the cell cycle phases of other polyphenic insects and in different polyphenic tissues after modification.
Protocol for Analyzing Cell Cycle Phases of Polyphenic Wings Using Flow Cytometry

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SUMMARY
Deciphering cell cycle phases of polyphenic tissues is an important challenge in understanding the cellular mechanism of polymorphism. We use flow cytometry to analyze cell cycle phases of short wings and long wings of the brown planthopper. This provides information on the arresting cell cycle phases in different wing forms. The protocol could be applied to analysis of the cell cycle phases of other polyphenic insects and in different polyphenic tissues after modification. For complete details on the use and execution of this protocol, please refer to Lin et al. (2020).

BEFORE YOU BEGIN
Insect

© Timing: 2 days

1. Prepare a batch of fifth-instar brown planthopper nymphs with rice seedlings (temperature: 25°C, relative humidity: 60%, photoperiod: 16 L: 8 D).

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, Peptides, and Recombinant Proteins | | |
| Triton X-100 | Sangon Biotech Co., Ltd. | A110694-0500 |
| DAPI Stain Solution | Sangon Biotech Co., Ltd. | E607303-0002 |
| KH₂PO₄ | Sangon Biotech Co., Ltd. | A501211-0500 |
| Na₂HPO₄ | Sangon Biotech Co., Ltd. | A501727-0500 |
| KCl | Sangon Biotech Co., Ltd. | A100395-0500 |
| NaCl | Sangon Biotech Co., Ltd. | A501218-0001 |
| Ethanol | Hangzhou Gaojing Fine Chemical Co., Ltd. | 23200151 |
| Cell Strainer, 40 μm | Sangon Biotech Co., Ltd. | F613461-9001 |
| Software and Algorithms | | |
| Flowjo | Tree Star | 7.3 |
| CytExpert | BECKMAN COULTER | 2.0 |

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Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Other               |        |            |
| Disposable tissue grinding pestle | Sangon Biotech Co., Ltd | F619072-0001 |
| Refrigerated centrifuge | HITACHI CENTRIFUGE | CT15RE |
| Flow cytometry      | BECKMAN COULTER | CytoFLEX |
| Fluorescent Microscope | NIKON    | ECLIPSE 80i |
| Experimental Models: Organisms/Strains | Brown planthopper, Nilaparvata lugens | n/a |

**MATERIALS AND EQUIPMENT**

**PBS Preparation**

| Name     | Final Concentration (mM) | Amount |
|----------|--------------------------|--------|
| KH₂PO₄   | 1.99                     | 0.27 g |
| Na₂HPO₄  | 10                       | 1.42 g |
| NaCl     | 136.80                   | 8 g    |
| KCl      | 2.68                     | 0.20 g |
| H₂O      | n/a                      | Add to 1 L |

**STEP-BY-STEP METHOD DETAILS**

### Dissection

**Timing:** 2 h

1. Pick the newly emerged long- and short-winged brown planthoppers, nymph undergoing the last molting and emerged. Make sure the brown planthopper is emerged in less than 5 min.
2. Place the insects in a Petri dish containing cold PBS. Under the stereoscope, use one tweezer to press against the abdomen of the brown planthopper, and the other removes wings and places them in a 1.5 mL centrifuge tube containing 200 µL PBS (Figure 1, Method Video S1).

**Note:** A pair of wings from one brown planthopper was put into a tube as one sample.

**CRITICAL:** keep the samples on ice. The processing time of this step should not be too long to prevent cell necrosis.

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*Figure 1. Remove Wings from Newly Emerged Brown Planthopper*
**Pre-treatment**

**Timing:** 4 h

3. **Tissue/cell separation:** grind the prepared sample with a grinding pestle.

   ▲ **CRITICAL:** Use a new grinding pestle for each sample. Do not reuse the pestle to grind the sample to prevent contamination. Samples should be ground sufficiently. The sample should be ground for at least 10 s. Insufficient grinding affects the collection of cells when the sample is loaded onto the machine.

4. **Cell sedimentation:** place the ground sample in a refrigerated centrifuge for centrifugation (10,000 × g, 4°C, 3 min).

5. **Cell fixation:**
   a. Discard the supernatant.
   b. Add 200 μL, 70% ethanol drop by drop (stored at −20°C).
   c. Place the sample on ice and fix for 1 h to ensure that the basic morphology of the cells does not change.

6. **Cell sedimentation:** Put the fixed sample into a refrigerated centrifuge and centrifuge for 3 min (10,000 × g, 4°C).

7. **Permeabilization step:**
   a. Discard the supernatant.
   b. Add 200 μL of PBS and 100 μL of 0.25% TritonX-100 respectively and mix well.
   c. Leave it at 4°C for 10 min.

   **Note:** A channel is formed in the surface of the cell to facilitate the subsequent entry of DAPI.

8. **Cell sedimentation:** put the sample into a refrigerated centrifuge and centrifuge for 3 min (10,000 × g, 4°C).

9. **Nucleic acid staining:**
   a. Discard the supernatant.
   b. Add 400 μL PBS and 2 μL DAPI fluorescent dye (excitation wavelength: 405 nm). (Troubleshooting 1)

   **Note:** DAPI binds strongly to DNA and emits fluorescence.

   c. Observe under a fluorescence microscope after 30 min in the dark. Then load the samples, the liquid ratio can be adjusted according to the number of samples.

**Cell Cycle Detection**

**Timing:** 3 h

10. **Before loading the samples,** filter the sample with a 40 μm cell strainer.

    **Note:** Prevent the sample from blocking the lines of the instrument. (Troubleshooting 2)

11. **Ready to use:**
    a. Check the sheath liquid barrel and waste liquid barrel to ensure that the liquid in both reservoirs is within the safe range.
    b. Turn on the flow cytometer and open the software.
    c. Start the flow cytometry process, use 2 mL of pure water to clean and preheat.
12. Name the sample, double-click the left button to set the channel used for the sample to PB450 (detection filter: 450 ± 45 nm), and select the appropriate channel according to the dyes used.

13. Set two dot plots (FSC-A, SSC-A), (PB450-A, PB450-H) and one bar graph (PB450-A, COUNT). Change the format of the graph to linear.
   a. Set the first scatter plot FSC-A/SSC-A to circle the cell population;
   b. Doublet discrimination should be performed. Set up a second scatter plot of PB450 height signal and area signal to remove doublets, and the cell population with the same area signal and height signal is a single cell population;
   c. Finally, the PB450 histogram is used to analyze the cycle status of the single cell population.

14. Sample loading, the number of collected particles is uniformly set at 3,000, which can be adjusted according to the number of effective particles. Save the data after the sample loading, and finally perform cleaning to end the program. (Troubleshooting 3, 4) (Figure 2).

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**Figure 2.** Representative Dot Plots Image of Cells from the Long and Short Wings and Flow Cytometry Analysis Histograms of Cell Cycle Phases

Upper: dot plots image of cells from the long and short wings and single cells were framed out; Lower: histograms shows the percentage each cell cycle phase of cells from the long and short wings.
Cell Cycle Analysis

© Timing: 2 h

15. Open the third-party software FlowJo and open the sample data in fcs format.
16. Identify valid cells:
   a. Set the first gate: (FSC-H, SSC-H) to delineate the cell population.
   b. Set a second gate: (PB450-A, FSC-W) to delineate the single cell population.

   Note: This step is to remove excessive cell debris.

   b. Set a second gate: (PB450-A, FSC-W) to delineate the single cell population.
17. Using “cell cycle” in “Tools” to quantify the percentage of G1, S, G2/M, and a histogram was added in FlowJo.

EXPECTED OUTCOMES

Given the cells in wing pads were arrested in different cell cycle phases at the end of development, and finally developed into long and short wings. Comparing cell cycle phases of the long and short wings of newly emerged adults can tell us whether they were arrested in different phases. Cell cytometry analysis showed that the cells of the long and short wings were arrested at G1 and G2/M phase separately (Figure 3).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data Collected

The independent sample t test is used to detect whether there is a difference between the two sets of data (Tables 1 and 2).

| Short-winged | 1  | 2  | 3  | 4  | 5  | 6  |
|--------------|----|----|----|----|----|----|
| G1           | 25.5| 21.2| 17.4| 23.1| 16.4| 20.6|
| S            | 4.8 |10.2| 13 | 9.7 | 8.8 |15.5|
| G2/M         | 69.7| 68.6|69.6| 67.2 | 74.8| 63.9|

Figure 3. Percentages of Cells in G1, S, and G2/M

G1 and G2/M differed significantly between cells from the short (n = 6) and long wings (n = 6). Student’s t test range-test was used for statistical comparison. n.s., not significant; **p < 0.001. Data are represented as means ± SD.
G1 Phase: G1 phase data of the long and short wings are compared using SPSS, and the first sig. >0.05 is found, indicating that the variance is homogeneous. The second sig. <0.001, indicating that there is a very significant difference between G1 of long and short wings (Table 3).

S Phase: Compare the data of Phase S between the long and short wings, and find that the first sig. >0.05, indicating that the variance is homogeneous. And the second sig. >0.05, indicating that there is no difference in the S phase of long and short wings (Table 4).

G2/M Phase: Compare the G2/M data of the long and short wings, and find that the first sig. <0.05, indicating that the variance is not homogeneous. The second sig. <0.001, indicating that there is a very significant difference between G2/M of long and short wings (Table 5).

**LIMITATIONS**

- **Collection of samples:** Collecting samples from different periods will produce different results, so the time for collecting samples must be controlled strictly.
- **Grinding time:** Long grinding time will cause the cells to break, and most of the cell debris will be collected when loading samples, which will affect the results. If the grinding time is too short, the cells will not be separated completely, and the cell mass will not be filtered through the cell strainer, resulting in insufficient particles.
TROUBLESHOOTING

Problem 1
How to select fluorescent dyes and channels/colors of flow cytometer model?

Potential Solution
Fluorescent dyes can be adjusted according to different flow cytometer models, and different fluorescent dyes use different channels. For example, the standard FACSCalibur has only one 488 nm laser (propidium iodide could be used as the DNA-binding dye), which can perform three fluorescent channels/three colors of FL1, FL2, FL3, and the optional Calibur (abbreviation of FACSCalibur) is equipped with two lasers of 488 nm and 635 nm, which can detect FL1 - FL4 four channels/four colors.

Problem 2
Samples block the lines of the instrument.

Potential Solution
Selection of cell strainer: Choose different cell strainer according to the cell size of the sample. If the cell volume is large, choose a cell strainer with a large mesh size. Otherwise, choose a strainer with a small mesh size.

Problem 3
Insufficient number of sample particles.

Potential Solution
When using flow cytometer, if the number of sample particles collected at low flow rate is too small, you can increase the amount of sample or adjust the ratio of reagent to sample. When discarding the supernatant, try not to touch the bottom and keep minimum amount of liquid to reduce the loss of the sample.

Problem 4
How to select liquid flow rate and injection speed?

Potential Solution
Selection of liquid flow rate when the machine is on: the lower the flow rate, the more accurate, but it will lead to a longer time. The injection speed should be selected reasonably.

RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Xinda Lin (linxinda@cjlu.edu.cn).

Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
This study did not generate any unique datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100080.
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AUTHOR CONTRIBUTIONS
X.L. conceived and designed the study, X.L. and Y.Z. performed experiments and analyzed data, X.L. wrote the paper. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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