Video Article

# Stomata Tape-Peel: An Improved Method for Guard Cell Sample Preparation

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## Abstract

The study of guard cells is essential to the knowledge of the specific contributions of this cell type to the overall function of plants. However, it is often difficult to isolate and thus studying them often provides a challenge.

This study establishes a stomatal guard cell enrichment method. The protocol utilizes Scotch tape to isolate guard cells and extract proteins from the cells. This method improves the integrity and yield of guard cell during isolation and provides a reliable sample for the study of cell signaling processes and how they correlate to stomatal movement phenotypes. In this method, the plant leaves were partitioned between two portions of tape and peeled apart to remove the abaxial side. This protocol was applied to Arabidopsis leaf tissue to isolate guard cells. The cells were treated with fluorescein diacetate (FDA) to determine viability and with abscisic acid (ABA) to assess stomata movement in response to ABA. In conclusion, this protocol proves useful for preparing enriched stomatal guard cells and isolating proteins. The quality of the cells and proteins obtained here enable physiological and other biological studies.

## Video Link

The video component of this article can be found at https://www.jove.com/video/57422/

## Introduction

Stomata play an important role in the overall physiology and fitness of plants. These tiny plant specific structures are formed by two specialized epidermal cells known as guard cells and are found most abundantly on the abaxial surface of leaves. Stomata are necessary for the exchange of gasses between the plant and the atmosphere, as well as the control of water loss through transpiration. Guard cells regulate stomata aperture through the integration of different external (*e.g.*, light, humidity, pathogen contact, carbon dioxide levels) and internal (*e.g.*, endogenous hormones) stimuli[1,2]. In the last 20 years, this cell type has become a model system for studying cell signaling processes in plants. These cells make up a small fraction of the total cells in a leaf; thus, to investigate their unique cellular, biochemical and molecular properties in a single-cell manner, it is necessary to isolate them from other leaf cell types. In the past, guard cell studies have often involved the preparation of guard cell protoplasts (GCP)[3,4,5]. This typically requires the use of large amounts of cell-wall degrading enzymes and or mechanical disruption via blending and has proven to be costly and time-consuming as it generally takes many hours to prepare a GCP sample, often times with little yield. More importantly, because guard cells act as complete pairs to regulate stomata aperture, the use of GCP can be seen as an artificial system to studying guard cell signaling.

Here, we have developed a method to prepare stomata in which intact guard cells are enriched and maintain physiological responses to stimuli. This method was inspired by a method that was used to isolate mesophyll cell protoplasts[6,7]. In our method, stomata are prepared using clear Scotch tape to first separate the majority of mesophyll cells attached to the adaxial layer of the leaf from the abaxial layer containing the pavement and guard cells. This is done by affixing a piece of tape to each side of the leaf and peeling them apart. The cells from the abaxial layer are allowed to recover under light in a stomata-opening buffer. Later the remaining pavement cells are removed using a small quantity of cell wall degrading enzymes, leaving behind guard cell that are enriched on the tape.

In this simple method, stomatal guard cells that are both viable and responsive can quickly and efficiently be prepared, taking less than an hour to obtain enriched stomatal guard cells from 50 peels with minimal cost. The method here imposes less damage to the plant cells than previous methods where protoplasts are prepared. In addition, materials collected from this method yield desirable protein amounts. Most importantly, because the leaf is not subjected to blending or lengthy digestion times and guard cells remain intact, the results of studies will more closely be relevant to that of a guard cell's natural biology. With this method, stomatal movements can be correlated in real time with changes

 

at the molecular level. Thus, the knowledge gained from studies using this preparation method would be important for a more comprehensive understanding of guard cell signaling.

We used the plant *Arabidopsis thaliana* as a model; however this protocol can be applied to the enrichment of stomatal guard cells in other plant species such as *Brassica napus* with small modifications in digestion time. As a proof of concept, we have demonstrated that stomatal guard cells enriched via this method are viable and responsive to stimuli as shown by the fluorescein diacetate (FDA) assay and studies utilizing the plant hormone abscisic acid (ABA), respectively. In addition, we have demonstrated that high-quality proteins can be isolated from these guard cells. Here, we describe a detailed protocol of this process.

## Protocol

# 1. Growing Plants

1. Germinate the seeds in potting mixture. Grow plants in a growth chamber with a light intensity of 140 µmol photons $m^{-2}$ $s^{-1}$ and a photoperiod of 8 h light at 22 °C and 16 h dark at 18 °C for 2 weeks.
2. Transplant seedlings of similar sizes individually into 4" diameter pots containing the soil and grow for an additional 3 weeks in the same conditions described in step 1.1.
   NOTE: Water plants with tap water twice a week to keep the soil moist.

# 2. Preparation of Enriched Stomata

1. Remove the leaves of 5-week old *A. thaliana* plants individual with a scalpel.
2. Attach each leaf to two pieces of clear Scotch tape, with one piece adhering to the abaxial (lower) side and the other piece adhering to adaxial (upper) side of the leaf. Leave the tape on the leaf for 5 s.
3. Gentle peel apart the two pieces of tapes using the index finger and thumb on each hand to separate the abaxial side with pavement and guard cells and the adaxial side with mesophyll cells.
4. Place the peels from the abaxial side of the leaves in 60 mL of stomata opening buffer (50 mM KCl, 10 mM MES-KOH, adjusted to pH 6.2 with 1 M KOH) in 40 mm × 12 mm size Petri dishes as they are collected.
   1. Repeat the above steps until all sample peels are collected.
5. Place the Petri dishes containing the peels into the growth chamber under light conditions stated in step 1.1. Leave the peels under the light for 2 h to fully open stomata.
6. Place peels into a 150 mm x 20 mm Petri dish containing 50 mL of cell wall digesting enzyme mixture (0.7% cellulase R-10, 0.025% macerozyme R-10 , 0.1% (w/v) polyvinylpyrrolidone-40, and 0.25% (w/v) bovine serum albumin in 55% basic solution (0.55 M sorbitol, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM ascorbic acid, 10 µM $KH_2PO_4$, 5 mM 4-morpholineethanesulfonic acid (MES) at pH 5.7 adjusted with 1 M KOH).
7. Shake the peels on a reciprocal shaker in a Petri dish at 50 rpm for 20 min.
8. Remove peels from enzyme solution with tweezers after 20 min. Transfer the peels to a 150 mm x 20 mm size Petri dish containing 50 mL of water.
9. Using a transfer pipette, rinse peels for 15 s twice with 10 mL of water to remove any residual enzyme solution.
10. Use tweezers to place peels in a 150 mm x 20 mm size Petri dish with 50 mL of fresh stomata opening buffer as used in step 2.4. Incubate for 1 h in light conditions stated in step 1.1 to allow the stomata to recover.

# 3. Abscisic Acid (ABA) Treatment and Stomata Movement Assay

1. In a 150 mm x 20 mm size Petri dish. Incubate the enriched stomata peels at 30°C for 15 min in 50 mL of the stomatal opening buffer or in 50 mL of the stomatal opening buffer with a final concentration of 10 µM ABA for the appropriate treatment time.
2. Place peels on a shaker at 50 rpm for 5, 15 and 30 min. After each time interval remove a peel with tweezers and follow the imaging steps in step 3.3.
3. Take images of stomata before ABA treatment and at the various time points after ABA treatment with a light microscope.
   1. Take a peel and place it on a glass microscope slide.
   2. Place the slide on the microscope stage and adjust the stage so that the image of the peel can be visualized.
   3. Adjust the fine and course focus on the microscope to get a clear image of the guard cells.
   4. Take several images of multiple stomata at 40X magnification.
4. Measure 60 stomatal apertures using ImageJ[8].
5. Calculate the standard error and significance at a p-value < 0.05 of the 60 stomatal aperture measurements.

# 4. Protein Extraction and SDS-Page Separation

1. Grind the peels for 15 s in enough liquid nitrogen to cover the peels using a chilled mortar and pestle.
2. Per 50 peels, add 3 mL of Tris saturated phenol (pH 8.8) and 3 mL protein extraction buffer (0.9 M sucrose, 0.1 M Tris-HCl, 0.01 M EDTA, 0.4% 2-Mercaptoethanol, 10 µL of 1 mM protease and phosphatase inhibitor) with a pipette to the mortar and then grind the peels for 5 additional minutes in a fume hood.
3. Transfer the extract using a pipette and the peels using metal tweezers to an Oakridge centrifuge tube and agitate on a shaker at 50 rpm for 1 h at 4 °C.

4. Remove the peels from the Oakridge centrifuge tube and centrifuge the extract at 5,000 x g for 10 min. Then transfer the top phenolic phase to a new clean micro-centrifuge tube using a pipette.
5. Precipitate the phenol extracted proteins by adding 5 volumes of ice-cold 0.1 M ammonium acetate in 100% methanol. Vortex briefly and incubate overnight at -20 °C.
6. Centrifuge at 20,000 x g at 4 °C for 20 min, decant the supernatant by slowly pouring it out of the tube and retain the protein pellet in the tube.
7. Wash the protein pellet twice with 0.1 M ammonium acetate in methanol, and then twice with 80% acetone and once with 100% cold acetone. NOTE: Washes are done by adding 10 mL of specified reagent to the tube of protein. Agitate on a shaker at 50 rpm for 5 min, followed by centrifugation at 15,000 x g at 4 °C for 10 min. Then pour the reagent out of the tube retaining only the protein pellet.
8. Add 1 mL of 100% cold acetone to the pellet and re-suspend by slowly pipetting up and down.
9. Transfer protein suspension via pipette to a 2 mL micro-centrifuge tube then centrifuge at 20,000 x g at 4 °C for 5 min.
10. Remove the acetone by decanting it from tube and dry the pellet in fume hood for 10 min.
11. Dissolve the protein in a micro-centrifuge tube with 200 µL of dissolution buffer (8 M urea, 0.5% SDS, 30 mM Tris-HCl at pH 8.5) and vortex for 30 min. Centrifuge at 20,000 x g at 15 °C for 20 min and collect the supernatant in a new tube.
12. Quantify the protein concentration following the protein quantification protocol[9].
13. Use protein samples for protein separation by gel electrophoresis following the protocol[10].

## Representative Results

A representative image of Arabidopsis guard cells before and after digestion of the mesophyll and epidermal cells is shown in **Figure 1**. Guard cell viability before and after the removal of mesophyll and epidermal cells can be observed using FDA to measure enzymatic activity and cell membrane integrity (**Figure 1B** and **1D**). **Figure 2** illustrates stomatal movement of Arabidopsis in response to ABA treatment. Stomatal aperture decreases more than 50 percent after 30 minutes of treatment with ABA. **Figure 2A** is a time course of stomata movement. At various time points, pictures were taken with a light microscope. Stomatal aperture was measured with ImageJ and results were presented as the average from 60 to 80 aperture measurements ± standard errors. A representative image of the stomata aperture at each time point is shown in **Figure 2B**. Protein detection, separation and relative abundance are observed by SDS-PAGE (**Figure 3**). Four biological replicates are included to emphasize the reproducibility of protein extraction from this method.

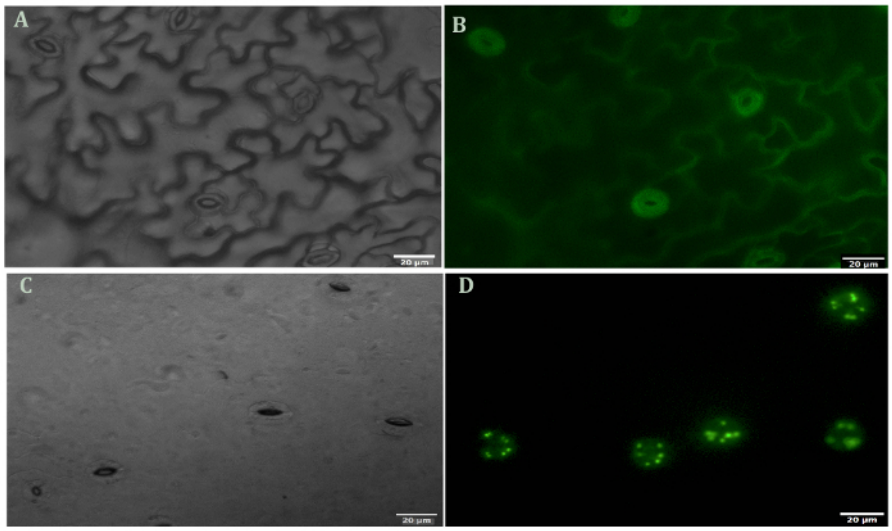


**Figure 1. Fluorescein diacetate viability staining of guard cells before and after the removal of mesophyll and epidermal cells. (A)** Peels (undigested) under bright field. **(B)** Peels (undigested) and stained with FDA. **(C)** Peels (digested) under bright field **(D)** Peels (digested) and stained with FDA. All pictures were taken at 40X magnification. Please click here to view a larger version of this figure.

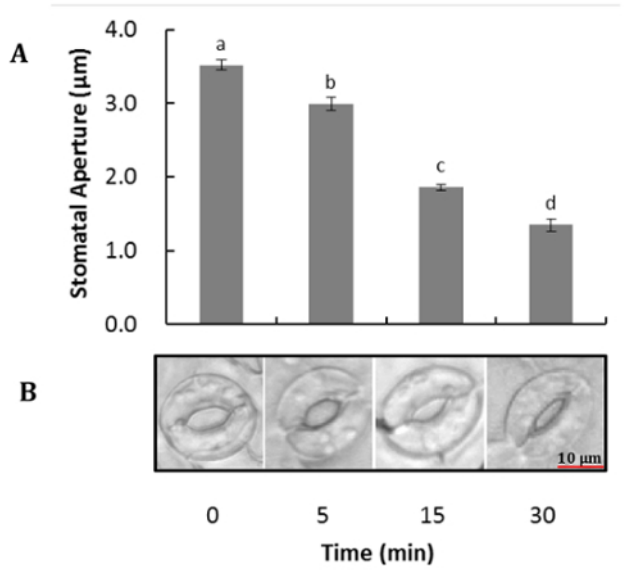


**Figure 2. Stomatal movement of Arabidopsis in response to ABA.** Epidermis were peeled from fully expanded leaves, with epidermal cells removed, and pre-incubated under light with the opening buffer (50 mM KCl, 10 mM MES-KOH, pH 6.2) for 2 h, and then incubated with water or with 10 μM ABA treatment for 5, 10 and 30 min. **(A)** Time course of stomatal movement. **(B)** Representative image of stomata at each time point. The data are shown as means ± SE of three independent experiments. Analysis of variance (ANOVA) was preformed to analyze mean differences. Different letters indicate significantly different mean values at $p<0.05$. Please click here to view a larger version of this figure.

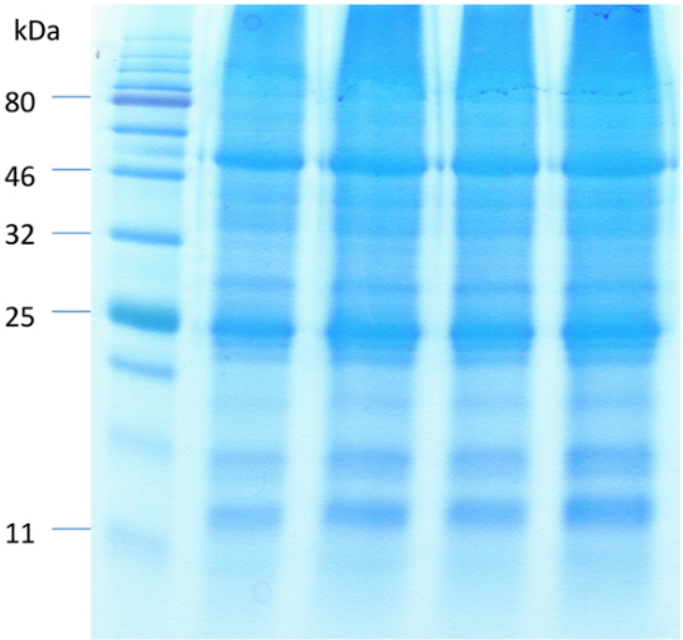


**Figure 3. SDS-PAGE separation of proteins from enriched guard cells using phenol-based protein extraction method.** Equal amounts (40 μg) of proteins were resolved using 12.5% polyacrylamide gel and visualized with CBB. Please click here to view a larger version of this figure.

## Discussion

Guard cells are a model system for studying signal transduction mechanisms in plants and it is important to ensure that the preparation of samples used in the study is the most appropriate to answering biological questions. Despite the increasing interest in guard cells within the plant research community, there is no universal method on how to prepare stomatal guard cells that would allow both the stomatal movement and physiology as well as the molecular changes to be studied in one system. The challenge to isolate them can be attributed in large part to their small size, low abundance and unique structure, which can make it more or less difficult to remove them from whole leaves.

This protocol provides a method on how to overcome these obstacles, isolating them using a developed tape-peel method. The method presented in this protocol provides a way for isolating intact stomatal guard cells, which remain physiologically responsive to stimuli. The

preparation of these samples makes it possible for the extraction of guard cell proteins that could serve as the starting material for in-depth proteomic studies, such as quantitative proteomic studies utilizing Tandem Mass Tags (TMT) and or Isobaric tag for relative and absolute quantitation (iTRAQ)[11,12]. In addition, this method can be slightly modified and optimized to make possible the study of small molecules, such as metabolites found in guard cells. The experiment here utilized the phytohormone ABA as an elicitor for stomatal movement in Arabidopsis, however other treatments may be used accordingly. This method is also adaptable to other plant species.

Due to the experimental design and the nature of materials used in this protocol, there are some critical steps. While peeling apart the abaxial and adaxial sides of the leaf (step 2.2), it is important that it is done without hesitation and by applying equal gentle pressure to both pieces of the tape so that the abaxial layer is uniform after it is removed. It is important to optimize individual peeling technique and digestion time for the plant species in the study as they may have different optimal digestion times. During the enzyme digestion (steps 2.8-2.10), the peels should be watched closely and checked every 3-5 minutes. This is important because slight differences in the layers of the peels will result in faster or slower digestion times. When treating the peels with ABA or other stimuli (step 3.1), peels should be equally spaced, floating and shaking gently so that they never overlap. Peels that get stuck together or overlap may yield different results due to lack of contact with solution.

## Disclosures

No conflicts of interest declared.

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