Identifying subcellular protein localization with fluorescent protein fusions after transient expression in onion epidermal cells

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Protein localization studies in onion epidermis

Summary

Most biochemical functions of plant cells are carried out by proteins which act at very specific places within these cells, for example within different organelles. Identifying the subcellular localization of proteins is therefore a useful tool to narrow down the possible functions that a novel or unknown protein may carry out. The discovery of genetically encoded fluorescent markers has made it possible to tag specific proteins and visualize them *in vivo* under a variety of conditions. This chapter describes a simple method to use transient expression of such fluorescently tagged proteins in onion epidermal cells to determine their subcellular localization relative to known markers.

Key words:

protein localization, transient expression, particle bombardment, fluorescence microscopy, onion epidermis, organelle markers

1. Introduction

Plant cells carry out a wide variety of functions, ranging from photosynthesis and basic metabolism over secretion and cytoplasmic streaming to environmental and pathogen responses. These functions depend on the proper distribution and interaction of a large number of proteins within the cells. In recent years, it has become evident that the dynamics of these protein distributions and interactions are essential for their function which makes it imperative to develop methods to identify these dynamic events in living cells.

Detection of subcellular localization and dynamics of proteins is usually achieved by creating genetically encoded fluorescent derivatives of the proteins of interest, for example by fusing them with green

fluorescent protein (GFP, Ref. ***1***). The discovery of red fluorescent proteins (***2***) combined with the targeted modification of fluorescent protein (FP) genes to create brighter varieties or different colors (e.g. Refs. ***3, 4***) allows for the direct comparison of two or more proteins within the same cell, thus greatly facilitating localization and protein-protein interaction studies. In fact, a number of collections of organelle marker constructs are available from stock centers (e.g. Refs. ***5, 6***) that make identification of subcellular localization studies relatively straightforward.

Stable transformation of plants with genes encoding fluorescently tagged proteins, preferably under control of their native promoters, is clearly desirable since this approach will allow for observation of long-term effects and, ideally, complementation of mutant phenotypes. This approach, however, requires a substantial investment of time. Transient expression approaches, on the other hand, can already yield important insights into protein localization and function and can be achieved without much technical effort by *Agrobacterium* infiltration (***7-9***). In this chapter, however, we describe the use of transient expression by means of particle bombardment. As long as the necessary equipment is available, particle bombardment is usually faster than agrobacterium-mediated approaches since it does not require integration of the gene construct into a binary plasmid and its mobilization into an appropriate *Agrobacterium* strain. The second part of the protocol describes basic epifluorescence techniques to visualize fluorescent proteins in living plant tissues which apply to all transformation techniques. The methods presented here can also be modified to accommodate more complex microscopy techniques such as laser scanning or spinning disk confocal microscopy.

2. Materials

2.1. Particle bombardment

This protocol is based on the PDS1000/He system (BioRad).

1. For onion tissues, M17 tungsten particles (1.0 µm, BioRad) work best.

2. A supply of macrocarriers, stopping screens, and rupture disks.

3. Agar plates with standard growth medium (for example, 1/2 x Murashige-Skoog medium, 1 % sucrose, pH6.0); one per sample.

4. 2.5 M $MgCl_2$.

5. 200 mM spermidine (store in -20°C freezer).

6. 70 % and 100 % ethanol.

7. Purified plasmids encoding the expression constructs (approx. 100 ng/µl). Miniprep DNA is usually sufficient.

8. Fresh onion.

9. Fine forceps;

10. Razor blades or scalpel.

2.2. Microscopy

1. Microscope slides and cover slips.

2. Overview objective (10x or 20x), high magnification objective (63x/1.4 NA, oil immersion).

3. Appropriate filters for fluorescence (see Table 1). As an alternative to the individual filter cubes for specific fluorescent proteins, a 'triple cube' (for example, Chroma set no. 69308) with separate excitation filters can be used. In this setup, specific fluorescent proteins can be excited by mounting the excitation filters in a separate filter wheel (e.g., Lambda 10-2, Sutter

Instruments) or a wavelength switcher (DG-4, Sutter Instruments) while the filter cube with the dichroic and emissions filters does not have to be changed. This setup allows for faster image capture but increases the risk of bleed-through between channels (see 3.7.). A conventional 'triple cube' that does not allow separate excitation of the fluorophores combined with a color camera is not advisable as it is virtually impossible to separate the different signals after capture for quantitative image analysis.

3. Methods

3.1. Preparation of particles

1. Weigh out 30 mg of M17 tungsten particles (1.0 µm; BioRad) in a microcentrifuge tube (**Note 1**) and add 500 µl 70 % ethanol (freshly prepared).

2. Vortex at half maximal speed for at least 10 minutes to suspend particles. Pellet particles in microcentrifuge for less than 5 s (**Note 2**).

3. Remove the supernatant with pipette and wash three times with 500 µl sterile water. At every wash step, vortex the particles for about one minute, let them settle for 1 min, and pellet them in a microfuge for less than 2 s (**Note 2**).

4. Resuspend particles in 500 µl sterile 50 % glycerol by vortexing. Particles are stable at room temperature for at least one month.

3.2. Preparation of onion tissue

1. Cut a fresh onion (**Note 3**) into quarters. Remove the innermost leaves since they are usually too highly curved.

2. Gently cut the epidermis on the adaxial (concave) surface into small strips of approximately 0.5 mm by 2 mm (**Note 4**).

3. Using fine forceps, peel off the epidermal strips and lay them, with the outer surface down, in the center of an agar plate. Collect enough epidermal strips to cover an area of about 3 cm in diameter. Prepare one petri dish per sample.

3.3. Preparation of macrocarriers

1. Combine expression plasmids in microfuge tube with a final volume of 10 µl (**Note 5**). Mix well.

2. Add 25 µl M17 particles from step 3.1 (thoroughly suspended by vortexing), 25 µl 2.5 M $MgCl_2$, 5 µl 200 mM spermidine.

3. Vortex mixture for 15 minutes at half-maximal speed. Let particles settle for 1 min before pelleting them for less than 2 s in a microcentrifuge (see **Note 2**).

4. Remove the supernatant without disturbing the pellet. Add 100 µl of 70 % ethanol (freshly prepared), wait 30 s, and remove supernatant (**Note 6**).

5. Repeat wash steps three times with 100 % ethanol to remove residual water.

6. Resuspend particles in 25 µl 100 % ethanol. Vortex particles for at least 1 min. Set pipettor to 15 µl and pipette particles up and down for a few times to break up larger clumps. Vortex particles again for 1 min.

7. Set down two macrocarrier disks (BioRad) on filter paper in an empty petri dish (**Note 7**). Place 8 µl of finely suspended particles into the center of each macrocarrier disk and put entire petri dish in 37°C incubator for 5 minutes to evaporate the ethanol (**Note 8**).

3.4. Bombardment

1. Turn on particle gun and vacuum pump.

2. Load rupture disk (1,100 psi; BioRad) and securely tighten holder (**Note 9**). Put stopping screen in macrocarrier assembly. Place the macrocarrier with the particles facing down in the holder and place on top of macrocarrier assembly. Slide macrocarrier assembly on top shelf.

3. Put open agar plate with onion peels on second shelf from the bottom.

4. Close particle gun and pull vacuum to about 27 mm Hg. Press "Fire" switch until rupture disk breaks. Vent the chamber and remove the agar plate.

5. Repeat the procedure (steps 2-4) with the same agar plate for the second macrocarrier disk of this sample.

6. After the second shot with the same sample, wrap agar plate with parafilm, and store in a dark place at room temperature until the next day (**Note 10**).

7. Repeat as needed for all samples.

3.5. Mounting epidermal peels for microscopy

1. Place clean coverslip on a paper towel and add small drops of water all over its surface.

2. With fine forceps, pick up the epidermal peel and place it on the cover slip in same orientation as on the agar (in other words, the outer surface that was down on the agar plate should also be down on the cover slip). Do this with a slight rolling movement (similar to rolling out a rug) to

prevent the formation of air bubbles between the coverslip and the tissue. Put as many epidermal strips on the cover slip as possible.

3. Put a few drops of water on the back of the epidermal peels. Gently lay a microscope slide on the coverslip and pick it up immediately; the cover slip will adhere to the slide.

3.6. Microscopy – identifying transformed cells

1. Orient yourself with a low magnification objective (10x or 20x) under brightfield illumination and focus on the epidermal cell layer (**Note 11**). Move sample so that the lower left corner of the tissue piece is in view.

2. Switch over to fluorescent illumination (**Note 12**) and scan over the tissue piece to identify transformed cells (**Note 13**). On an inverted microscope, the position of transformed cells can be marked on the upper side of the slide with a small dot from a felt-tip marker to make it easier to return to them later. On upright microscopes, the coordinates can be noted down from the stage markings (**Note 14**).

3.7. Microscopy – high magnification imaging

1. Switch to a high-magnification objective suitable for observation of subcellular structures (e.g., 63x/1.4 oil immersion). Focus on the marker signal. The highest quality images can be obtained in the cytoplasm right behind the outer plasma membrane (**Note 15**). This area also has the advantage that many organelles can be observed at the same time.

2. Switch the fluorescent filters to observe the signal from the unknown protein. Ideally, the signal of the marker and the unknown should be of roughly equal intensity to avoid bleed-through (**Note**

16). As a general rule, the exposure setting should be adjusted such that the signal brightness becomes maximal without saturating any pixels (**Note 17**). Since most organelles show rapid movements, it may be necessary to limit the exposure time to prevent streaking of the organelles (**Note 18**). The two images for the unknown protein and the organelle marker have to be taken in close succession to minimize movement of the organelles between images (**Note 19**).

3. To remove background noise, a second set of images should be taken with the same exposure settings but the excitation light shutter closed (**Note 20**).

3.8. Image analysis

After background subtraction (see **Note 20**) the images can be analyzed. To detect colocalization, the two images can be false-colored and superimposed to reveal overlapping signals. The best colors to use are red and green since their overlap will result in a different color, yellow, that can easily be detected (**Note 21**). This can be achieved by creating a new RGB image in ImageJ, converting this into an RGB stack (with "Image>Type") and pasting the two images into the first and second frame of the stack. Converting this image back to an RGB image will complete the procedure.

4. Notes

1. The tungsten particles can be hazardous because of their small size. Wear gloves and possibly also a respirator.

2. Keep the centrifugal force to a minimum to prevent clumping of the particles since this makes it difficult to resuspend them later.

3. Commercial onions usually work well. Avoid previously frozen onions and do not keep cut onions more than a few days.

4. The smaller size of the strips makes it easier to spread the curved epidermal peels on the flat agar surface.

5. Anywhere from 100 ng to 1 µg of DNA per plasmid is usually sufficient for clearly detectable expression of fusion proteins. The precise amount depends on the size of the plasmid, the promoter and nature of the fusion protein. Organelle markers tend to express very well and require little DNA (50 – 100 ng).

6. There is no need to resuspend the particles by vortexing. Several tubes can be prepared in parallel.

7. There should be enough particles for 3 disks, but two shots per sample normally yield sufficient numbers of cells to determine protein localization. The second shot can be omitted too, but is handy if something goes wrong with the first.

8. The particles should be finely dispersed on the macrocarrier. Clumps will lead to cell death and lower transformation rates. Presence of small amounts of water in the resuspended particles will lead to clumping during drying. It is not necessary to put the macrocarriers in a 37°C incubator when the relative humidity in the lab is low.

9. If the rupture disk holder is not securely tightened, the disk will slip out at low pressure. This may still result in transformation, but the yield of expressing cells may be lower.

10. Optimal incubation time depends on the proteins that need to be expressed. First signals can usually be observed after a few hours. Expression can be stable for several days, but fungal growth often appears on the second day of incubations. A typical incubation is between 16 and 24 h, in other words from the afternoon of one day to the morning or afternoon of the next.

11. The epidermal cells can be identified by their well-defined outlines. Further into the sample are the mesophyll cells which were typically broken during the peeling process. It is important that the epidermis is closest to the cover slip to ensure best image quality.

12. It is usually best to perform the initial scan with illumination for an organelle marker since they tend to express very well and result in bright fluorescent images. For example, a CFP marker is easy to detect while the weak autofluorescence of the cell walls will allow for orientation during the scanning process. Scanning for RFP is usually more difficult since the tissue emits very little signal at this wavelength and makes orientation difficult. Autofluorescence of the tissue, e.g. resulting from damage during preparation, can sometimes be confused with actual signal from the FPs. However, most autofluorescence appears with several fluorescence filters whereas FP fluorescence can be detected only by the appropriate filter set.

13. It is best to follow a regular path to ensure that all cells are examined. For example, move the stage up in a straight line to observe the left edge of the sample, shift over to the right by approximately one field of view, and move down until the lower edge of the tissue is reached, etc.

14. In most cases, distribution of transformed cells is not uniform but occurs in patches. It is often possible to identify patches with ten to twenty or more cells in a small area. These patches are very convenient since it is easy to move to neighboring cells even with a high-magnification objective.

15. Depending on the intracellular distribution of the fluorescent signal, it may be necessary to focus further into the cell. For example, the nucleus of these epidermal cells is usually found attached to the back wall, about 70 µm into the cell. Due to diffraction of light by the anticlinal walls (parallel to the incident light), however, the image quality in this part of the cell is not as high as closer to the cover slip.

16. FPs have both broad absorption and emission peaks. As a result, we often observe bleed-through of one fluorophore into the filter set of another fluorophore. For example, GFP will be visible with both CFP and YFP filters. Similarly, it is possible to excite RFP with GFP or YFP filters. This problem becomes more noticeable with multi-wavelength filters, but it can also occur with dedicated filter sets when one fluorophore is very bright and the other is very dim. In this case, the long exposure times necessary to detect the weak second signal may make it possible to pick up the small amount of bleed-through of the brighter fluorophore. To test whether this problem may occur, perform a transient expression with a single marker only and collect an image with optimal settings for this fluorophore. Then switch to another filter set and take an image with identical settings. Repeat taking images with increasing exposure times (twice as long, four times as long, etc.) until the signal becomes visible with this "wrong" filter. Depending on the filters and the fluorophores, a two-to-four-fold difference in exposure time may result in negligible bleed-through, but this has to be determined for every microscope setup independently. Also note, that contrast enhancement may bring out weak signals that may go unnoticed at first. Once these "bleed-through limits" have been established, it is possible to detect this problem simply by comparing exposure settings for marker and unknown. Should this be a problem, it may be possible to reduce the amount of DNA for the marker construct to reduce its signal.

17. The maximal signal intensities per image pixel should be about 10 % below the maximum that can be handled by the camera. This would be an intensity of about 3700 for 12-bit cameras, 15,000 for 14-bit cameras, and 59,000 for 16-bit cameras. Bright signals ensure highest signal-to-noise ratios and hence best image quality.

18. Organelles can often move with speeds exceeding 2 µm/s and reaching as high as 8 or 10 µm/s. In this case, it is necessary to reduce exposure times to 250 ms or shorter. This may require increasing the electronic gain setting of the camera, even though this tends to increase the noise more than longer exposure times.

19. If possible, computer automation should be used to capture the two images in close succession. Typically, the exposure settings are first determined and stored in the computer which then controls the microscope to expose the camera with the appropriate filter sets. With this approach, the delay between the images is only limited by the exposure times and the speed with which the filter sets can be switched. For this reason, a multi-wavelength filter cube combined with an external excitation filter switcher (see 2.2) is of advantage. In this case, the chance the bleed-through between channels is increased (see **Note 16**).

20. All camera images bring a certain level of background noise with them, which is evident in pixel values significantly above 0. For quantitative image analysis, it is necessary to remove this background in order to obtain accurate measurements of fluorescent signal intensity. The simplest way to achieve this is to subtract the pixel intensities of the background images from the experimental images. Most microscope software programs have this command built-in. The same effect can be achieved with the "Process>Image Calculator…" command in ImageJ.

21. Journals often recommend using magenta and green to accommodate red-green colorblind people. In this case, the red channel is duplicated in the blue channel which is visible to colorblind people. While this often achieves the same effect as red-green images, it tends to be more difficult to identify weakly colocalizing signals. Under no circumstances should the "true" colors be used, since the overlap, for example, of cyan (for CFP) and yellowish-green (for YFP) does not lead to a distinct signal and therefore cannot be clearly distinguished from the individual channels.

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Table 1: Filters recommended for visualizing common fluorescent proteins.

Fluorescent protein	**Excitation**	**Dichroic**	**Emission**
CFP	BP 436/25*	455	BP 480/40
GFP	BP 470/40	495	BP 525/50
YFP	BP 500/25	515	BP 535/30
RFP (mCherry)	BP 572/25	590	BP 629/62
Triple cube (CFP + YFP + mCherry)	BP 430/24	multiple transmission windows	TBP 470/24
	BP 500/20		TBP537/30
	BP 577/25		TBP635/65

*BP 436/25 = bandpass filter centered around 436 nm with a transmission window width of 25 nm at half-maximal height.