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Video Article

Cell Specific Analysis of *Arabidopsis* Leaves Using Fluorescence Activated Cell Sorting

Jesper T. Grønlund[1], Alison Eyres[1], Sanjeev Kumar[1], Vicky Buchanan-Wollaston[1,2], Miriam L. Gifford[1,2]

[1]School of Life Sciences, University of Warwick

[2]Warwick Systems Biology, University of Warwick

Correspondence to: Jesper T. Grønlund at j.t.gronlund@warwick.ac.uk

Abstract

After initiation of the leaf primordium, biomass accumulation is controlled mainly by cell proliferation and expansion in the leaves[1]. However, the *Arabidopsis* leaf is a complex organ made up of many different cell types and several structures. At the same time, the growing leaf contains cells at different stages of development, with the cells furthest from the petiole being the first to stop expanding and undergo senescence[1]. Different cells within the leaf are therefore dividing, elongating or differentiating; active, stressed or dead; and/or responding to stimuli in sub-sets of their cellular type at any one time. This makes genomic study of the leaf challenging: for example when analyzing expression data from whole leaves, signals from genetic networks operating in distinct cellular response zones or cell types will be confounded, resulting in an inaccurate profile being generated.

To address this, several methods have been described which enable studies of cell specific gene expression. These include laser-capture microdissection (LCM)[2] or GFP expressing plants used for protoplast generation and subsequent fluorescence activated cell sorting (FACS)[3,4], the recently described INTACT system for nuclear precipitation[5] and immunoprecipitation of polysomes[6].

FACS has been successfully used for a number of studies, including showing that the cell identity and distance from the root tip had a significant effect on the expression profiles of a large number of genes[3,7]. FACS of GFP lines have also been used to demonstrate cell-specific transcriptional regulation during root nitrogen responses and lateral root development[8], salt stress[9] auxin distribution in the root[10] and to create a gene expression map of the *Arabidopsis* shoot apical meristem[11]. Although FACS has previously been used to sort *Arabidopsis* leaf derived protoplasts based on autofluorescence[12,13], so far the use of FACS on *Arabidopsis* lines expressing GFP in the leaves has been very limited[4]. In the following protocol we describe a method for obtaining *Arabidopsis* leaf protoplasts that are compatible with FACS while minimizing the impact of the protoplast generation regime. We demonstrate the method using the KC464 *Arabidopsis* line, which express GFP in the adaxial epidermis[14], the KC274 line, which express GFP in the vascular tissue[14] and the TP382 *Arabidopsis* line, which express a double GFP construct linked to a nuclear localization signal in the guard cells (data not shown; **Figure 2**). We are currently using this method to study both cell-type specific expression during development and stress, as well as heterogeneous cell populations at various stages of senescence.

Video Link

The video component of this article can be found at http://www.jove.com/video/4214/

Protocol

1. Protoplast Generation

1. Harvest leaf material and cut into 1 mm wide strips (**Figure 1**) with a razor blade. For samples containing more than a single leaf up to 15 leaves can be easily stacked before cutting.
2. Immediately transfer leaf strips into a 9 cm round Petri dish containing 20 ml **solution A** (400 mM mannitol, 20 mM MES hydrate, 20 mM KCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1% BSA and 2.5 mM β-mercaptoethanol, pH 5.7) including protoplasting enzymes (1.2% cellulose R-10, 0.6% cellulose RS and 0.4% macerozyme R-10) and RNase and transcriptional inhibitors (1×ProtectRNA and 10 μg/ml Actinomycin D); gently separate the leaf strips and vacuum infiltrate for 2 × 5 min.
3. Place Petri dish on an orbital shaker set to 90 rpm at room temperature and protoplast for 3-4 hr.

1. During the incubation the leaf strips should be separated from each other, by use of a set of flat tweezers, at half hourly intervals.

4. Place a 70 µm cell strainer (Fisher Scientific) in a 50 ml tube and gently filter the protoplasting solution through. This will remove the leaf strips, which will be retained by the sieve while allowing the protoplasts to go through.
5. Wash the leaf strips with 4 ml **solution A** and gently filter through the same sieve.
6. Transfer the protoplasts into round bottom tubes and centrifuge the protoplast solution at 300 x g for 5 min to pellet the protoplasts.
7. Gently aspirate off as much of the supernatant as possible without disturbing the protoplasts.
8. Wash the protoplasts with 5 ml **solution A** and centrifuge at 300 x g for 5 min. Remove supernatant
9. Resuspend the protoplasts in an appropriate volume of **solution A**, using a Pasteur pipette or a cut-off P1000 tip.

2. FACS of Leaf Protoplasts

1. Immediately before sorting, resuspend the protoplasts using a (non-cut) P1000 tip to avoid protoplasts clumping together.
2. Transfer protoplasts to a sorting tube through a 50 µm filcon filter (BD Biosciences) and dilute as appropriate to avoid clogging the nozzle of the cell sorter. The protoplasts are sorted using a 100 µm nozzle at a pressure of 20 psi (sheath) and 21-21.5 psi (sample), a frequency of 39.2 kHz and an event rate of <4000 (these settings are optimal on a BD Influx cell sorter (BD Biosciences). Optimization on other cell sorting machines may need adjustments to these settings).
3. GFP positive protoplasts are identified using a 488 nm argon laser and plotting the output from the 580/30 bandpass filter (orange) vs. the 530/40 bandpass filter (green). GFP positive protoplasts will be in the high 530/low 580 population, with non-GFP protoplasts in the low 530/low 580 population and dead/dying protoplasts and debris in the high 580 population (**Figure 2**).
    1. When sorting protoplasts from which RNA will be extracted, protoplasts should be sorted directly into at least 4 volumes of a lysis buffer containing a reducing agent (*e.g.* β-mercaptoethanol). In addition, the sort time of a single sample should be limited to 20-30 min, and a maximum of two to three samples processed at any one time.
    2. For visualization of sorted protoplasts, the protoplasts should be sorted directly into at least 8 volumes of **solution A** and subsequently collected by centrifugation to concentrate the protoplasts.

3. Representative Results

This protocol describes a method for obtaining protoplasts from *Arabidopsis* leaves, which are used for fluorescence activated cell sorting (**Figure 1**). Protoplast generation in the presence of RNase and transcriptional inhibitors prevents the cells from mounting a transcriptional response. This does have the consequence of giving rise to many dead and dying protoplasts, which have a significant level of autofluorescence. When sorting the protoplasts prepared with this method it is therefore very common to see several distinct populations in graphs of the 580 nm vs. 530 nm fluorescence used for the sorting. Examples of this, together with the sorting gates used in this protocol are shown in **Figure 3**.

Protoplasts can be sorted into 8 volumes of solution A and collected for visualization to verify the enrichment. **Figure 2G** shows an example of this using the TP382 line, which expresses GFP in the guard cells.

Examples of yields obtained using this method, both before and after amplification, are shown in **Table 1**. The amounts obtained are sufficient for amplification using the Ovation Pico WTA System (500 pg-50 ng) and therefore subsequent analysis by either qPCR (**Figure 4**), next generation sequencing or microarray.
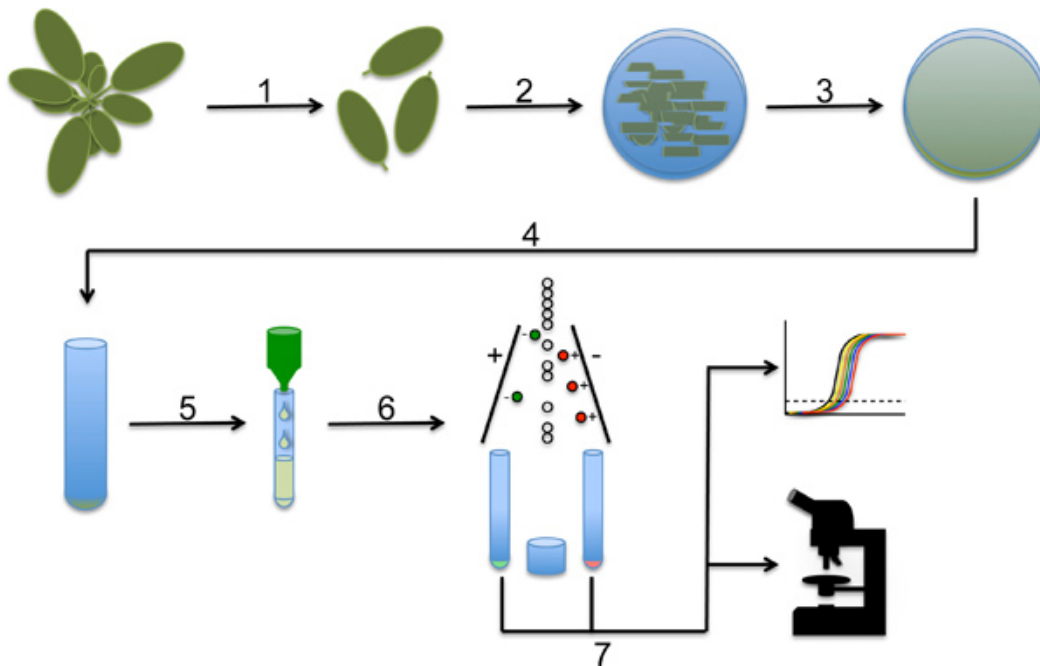
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Figure 1. Overall workflow for the experiment. 1) Harvest leaves of interest from a GFP expressing *Arabidopsis* line. 2) Quickly cut into ~1 mm wide strips and immediately transfer these into protoplasting solution and vacuum infiltrate. 3) Incubate for 3-4 hr. 4) Gently filtrate protoplast solution through a 70 µm sieve, transfer to round bottom tubes and pellet protoplasts by centrifugation. 5) Immediately before sorting, resuspend protoplasts and transfer to a sorting tube through a 50 µm filter. 6) Sort GFP positive and GFP negative viable protoplasts. 7) Protoplasts can now be analyzed either visually or using techniques such as qPCR, next generation sequencing or microarray.
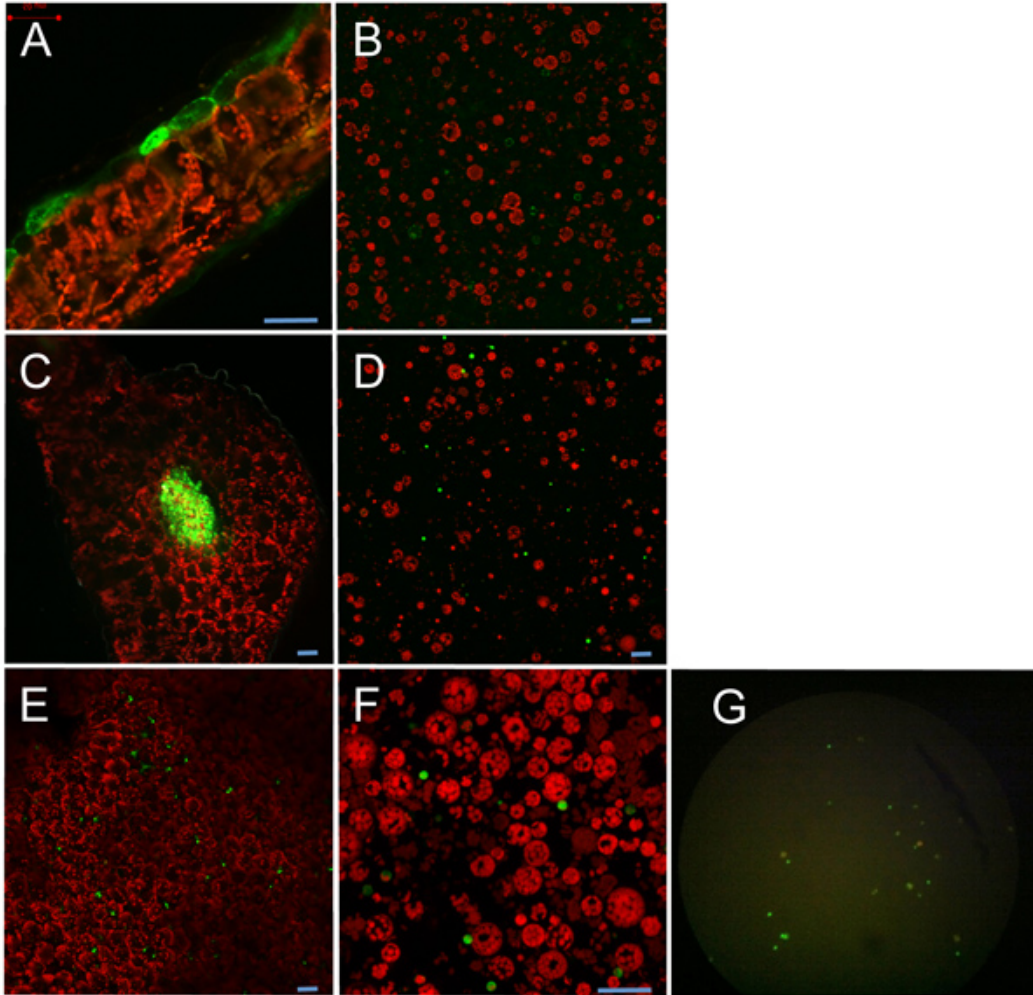
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Figure 2. The KC464, KC274 and TP382 lines used in this study. A) Transverse section of a KC464 leaf showing GFP expression in the adaxial epidermis. B) Protoplasts derived from KC464 leaves. C) Transverse section of a KC274 leaf showing GFP expression in the vasculature. D) Protoplasts derived from KC274 leaves. E) TP382 leaf showing GFP expression in the guard cells. F) Protoplasts derived from TP382 leaves. G) Example of the enrichment obtained by FACS of GFP expressing guard cells in the complex background of leaf cells, showing GFP containing protoplasts from the high 530/low 580 population of protoplasts derived from TP382 leaves. A-F: Confocal microscope images; G: Fluorescence microscope image. Scale bars within images are 50 μm.
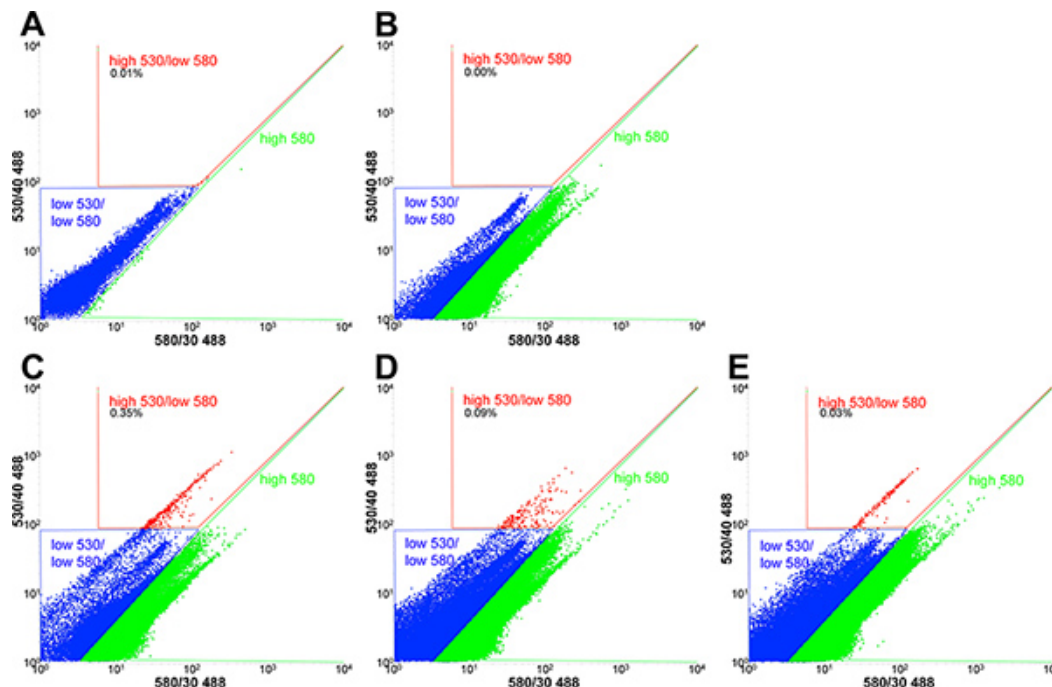
Figure 3. Typical FACS acquisition dot plots of the output from the 580/30 nm (orange) vs. 530/40 nm (green) bandpass filters (BD Influx cell sorter). The sorting gates shown are those typically used during sorting. A) Dot plot of Col-4 leaf derived protoplasts, protoplasted in the absence of inhibitors, showing a single low 530/low 580 population. B) Dot plot from wt Col-4 leaf derived protoplasts, protoplasted in the presence of inhibitors, showing a shift in the 580 fluorescence due to stress and coloring by the inhibitors. C-E) Dot plots from leaf derived protoplasts from the KC74, KC464 and TP382 lines, respectively, protoplasted in the presence of inhibitors, showing the GFP containing populations in the high 530/low 580 populations. The percentages of the events in the GFP (high 530/low 580) populations are given. Click here to view larger figure.
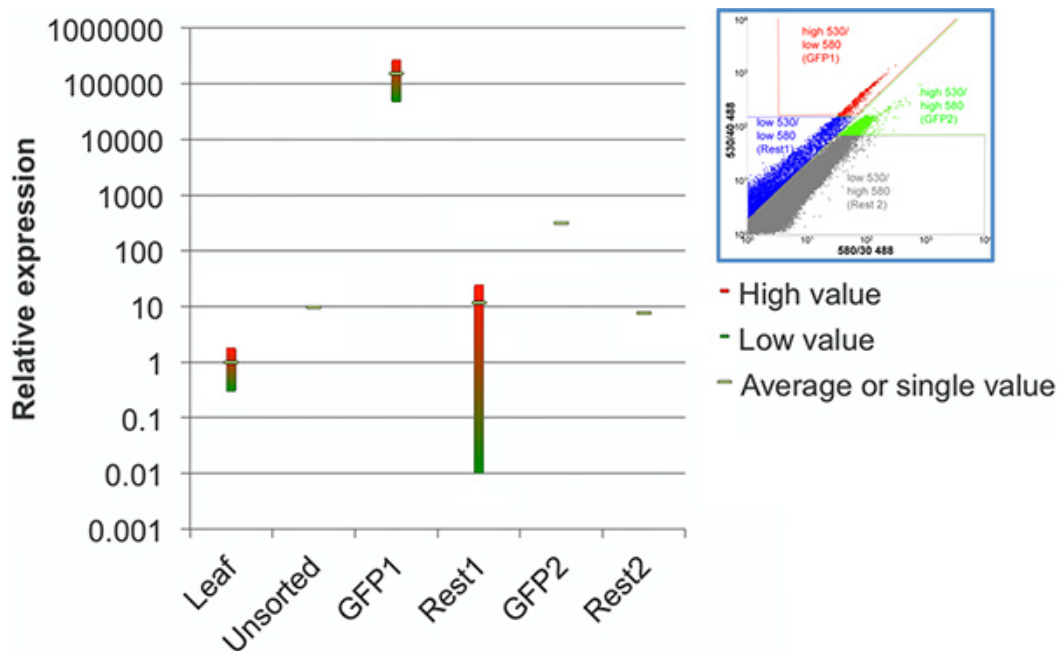

Figure 4. GFP expression in the representative gated population fractions as shown in **Figure 3**. Two biological replicates were obtained for each fraction type, amplified using the Ovation Pico WTA System and qPCR using GFP-specific primers (**Table 2**) was then performed. Values shown are the highest (red), lowest (green) and average relative values. The high 580 population was in this case split in two, low 530/low 580 and high 530/low 580, for which a single biological replicate was used so only the single value is shown. ACT2 (At3g18780) was used as a standard for all samples. Leaf: Whole leaf. Unsorted: Unsorted protoplasts. GFP1: high 530/low 580 and Rest1: low 530/low 580, GFP2: high 530/high 580 and Rest2: low 530/high 580, as described in **Figure 3** and the insert. The results are a verification of optical assessments of GFP cell content in the sorted fractions (*e.g.* **Figure 2G**). Click here to view larger figure.

Sample	No. of leaves	No. of cells	RNA yield (ng)	RNA yield (amplified; ng)
Whole leaf A	1	-	8001	4623***
Whole leaf B	1	-	10525	4572***
Unsorted protoplasts A	5	-	2433	1152***
Unsorted protoplasts B	5	-	2625	3321***
GFP1 A	15	6039 (0.39%*)	-**	861
Rest1 A		55389 (6.40%*)	-**	489
GFP1 B	15	10783 (0.63%*)	-**	537
Rest1 B		57040 (7.49%*)	-**	420
GFP2	15	18337 (1.49%*)	-**	360
Rest2		62405 (73.49%*)	-**	411

Table 1. Samples collected using FACS and analysed with qPCR to determine which fraction contained live GFP-expressing protoplasts. Also included in this table is the total RNA yield in ng before and after amplification with the Ovation Pico WTA System (NuGen). GFP1, GFP2, Rest1 and Rest2 fractions are as shown in the insert in **Figure 4**. *The percentage of cells of the total cell sort run is given in parentheses beside the number of cells in the fraction. These percentages are not correlated to each other for each sample as the FACS sort runs for 'Rest' fractions were stopped earlier than for GFP fractions due to the much greater number of Rest cells collected. **50 ng used as input for amplification reactions, as per manufacturer's protocol.

Primer	Sequence
mGFP5-ER.fw	GCCTGAGGGATACGTGCAGGAGA
mGFP5-ER.rv	TGGCGGGTCTTGAAGTTGGCT
ACT2.fw	GCCATCCAAGCTGTTCTCTC
ACT2.rv	GCCATCCAAGCTGTTCTCTC

Table 2. qPCR primers.

Discussion

We have described a method that allows the use of *Arabidopsis* plants expressing GFP in the leaves for protoplast generation and subsequent cell sorting using FACS. This method yields sufficient amounts of RNA to be used for amplification and therefore subsequent analysis by either qPCR or microarray. The fractions sorted are highly enriched for GFP-containing cells, as demonstrated by qPCR (**Figure 2**).

To achieve high yields of live protoplasts it is critical to work rapidly during the initial steps of the procedure, until the leaf strips have been vacuum infiltrated with the inhibitor-containing protoplast solution. In addition, when generating 1 mm leaf strips it is very important to slice or cut the leaves rather than 'chop' or mash them, as this leads to excessive damage and thus a lower protoplast yield.

A critical difference between the method described here and other published methods is the use of RNase inhibitors and transcriptional inhibitors. Most methods are developed for *Arabidopsis* roots, from which protoplasts can be generated more easily. However, when working with leaves, excepting mesophyll, it is necessary to increase the protoplast generation time. Therefore it is important to include these inhibitors to protect against changes in gene expression as a result of the protoplast generation treatment itself (data not shown). However, the presence of the inhibitors leads to an inability to mount a transcription response, either by switching genes on or off, which is likely to make the protoplasts more vulnerable as this leads to a loss of plasticity with which to counter the stresses of the protoplasting procedure.

We have used the method described here successfully with a number of GFP- expressing *Arabidopsis* lines. Importantly, we have used this method with GFP lines expressing GFP either strongly in the nucleus, or much more weakly in a non-localized/diffuse cytosolic manner, as is the case with the KC464 and KC274 lines used here. Both types of lines are compatible with the method and yield highly enriched fractions of GFP-expressing protoplasts (**Figure 2 and 3**). The method could be easily adapted to other fluorophores, although the fluorophore must be selected for fluorescence that is significantly different from the autofluorescence of dead/dying cells in order to preserve the ability to select live cells.

Disclosures

No conflicts of interest declared.

Acknowledgements

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