Automating measurements of fluorescent signals in freely moving plant leaf specimens

Natsuko Kinoshita[1,*], Aki Sugita[1,2,†], Barry Lustig[3,†], Shigeyuki Betsuyaku[1],
Tetsu Fujikawa[4], Tatsuji Morishita[5]

[1] Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan;
[2] Department of Evolutionary Studies of Biosystems, School of Advanced Sciences, SOKENDAI (The Graduate University for Advanced Studies), Shonan Village, Hayama, Kanagawa 240-0193, Japan; [3] Cormorant Group, Pittsburgh, Pennsylvania 15243, USA; [4] Aoba, Yokohama, Kanagawa 227-0051, Japan; [5] Leica Microsystems, 1-29-9 Takatanobaba, Shinjuku, Tokyo 169-0075, Japan
* E-mail: kinoshita.natsuko.gf@u.tsukuba.ac.jp   Tel: +81-29-853-4798   Fax: +81-29-853-6617

Abstract   Existing methods to quantify fluorescent signals are primarily limited to non-moving objects or tracking a limited number of cells. These techniques, however, are unsuitable for measuring fluorescent signals in time-lapse experiments using plant specimens that move naturally during a course of imaging. We developed an automated method to measure fluorescent signal intensities in transgenic Arabidopsis plants using a stereomicroscope with standard microscopy software. The features of our technique include: 1) recognizing the shape of plant specimens using autofluorescent signals; 2) merging targeted fluorescent signals to specimen outlines; 3) extracting signals within the shape of specimens from their background signals. Our method facilitates the measurement of fluorescent signals on freely moving plant leaves that are physically unrestrained. The method we developed addresses the challenge of recognizing plant shapes without relying on: a) manual definition which is prone to subjectivity and human error; b) introducing stable fluorescent markers to define plant shapes; c) recognizing plant shapes from bright field images which include a wide range of colors and background noise; d) unnecessarily stressing plants by immobilizing them; e) the use of multiple software packages or software development expertise.

Key words: autofluorescence, fluorescence, shape recognition, time-lapsed, tracking.

Introduction

Over the past decade, there has been a rapid development of bio-imaging tools to monitor gene expressions as well as chemical compound levels in plant cells (Grossmann et al. 2018; Jones et al. 2014; Rizza et al. 2017; Waadt et al. 2014).

Manually defining Region of Interest (ROI) is the simplest way to analyze gene expressions in time-lapsed experiments using freely moving leaves. Allowing plant leaves to move freely better simulates a natural setting than immobilizing specimens.

While manual definition of ROI is still considered the gold standard (Khushi et al. 2017), it is prone to human error. For instance, Huth et al. (2010) reported that the rate of human error when manually tracking ROI in pancreatic cells was as high as 410%.

Larrieu et al. (2015) visualized the activation of jasmonic acid signaling pathways using a biosensor. To aid quantification, they used double transgenic lines with a constitutively expressed nuclear fluorescent marker (Histone H2B). Rellán-Álvarez et al. (2015) developed imaging platform to visualize root architecture and gene expression, accompanied with GLO-RIA, a specialized software system to analyze root system properties and their dual reporter images.

We sought to develop an automated method to track plant shapes using autofluorescence which neither required a transgene of a constitutive marker nor multiple software packages.

Materials and methods

Plant materials

The transgenic Arabidopsis line consisted of 3.0 kb of the promoter region from *VEGETATIVE STORAGE PROTEIN1* (*VSP1*) which was fused to *YELLOW FLUORESCENT PROTEIN* (*YFP*) with a *NUCLEAR LOCALIZATION SIGNAL* (*NLS*) using a Columbia-0 background (Betsuyaku et al. 2018).

Growth conditions

Transgenic Arabidopsis seeds were sterilized with bleach. The seeds were then sown on Murashige and Skoog media, which contained 0.8% agar (Difco) and 1% sucrose (Murashige and Skoog 1962). The plants were grown in a standard condition of 16 h light/8 h dark cycles at 22°C for three weeks.

Treatment

Methyl dihydrojasmonate (Tokyo Kasei) was diluted to 5 mM in dichloromethane and 20 μl was applied to a cotton ball (Bodnaryk and Yoshihara 1995; Kishimoto et al. 2006; Rohloff and Bones 2005). The cotton ball was then placed on a plastic support on Murashige and Skoog media to evaporate the methyl dihydrojasmonate.

Time-lapse imaging

To acquire live images, we used a M205FA automated stereomicroscope with a motorized stage. Images were taken with DFC7000T color CCD camera (Leica Microsystems). This apparatus was controlled by LasX software (Leica Microsystems). A metal halide bulb (Leica EL6000) was used for excitation light source. Chlorophyll autofluorescence (Komis et al. 2015) and YFP signals were detected by Texas Red and YFP filters, respectively (both from Leica Microsystems; Kinoshita and Betsuyaku 2018).

Excitation emission wavelengths of YFP and Texas Red filters were 510/20–560/40 nm and 560/40–610 LP nm respectively. The Texas Red filter reduced nearly all of the nonspecific autofluorescent signals from dead plant cells (Betsuyaku et al. 2018).

The bright field, YFP and Texas Red images were captured every 20 min. Plant specimens were exposed to white light during these intervals using LasX software (Leica Microsystems).

Data analysis

I) Manual measurements using FIJI software

We measured every eighth frame of the 70 frames recorded during a 24-hour period (there are 70, 20-min intervals within 24 h) to analyze YFP intensity manually. Captured bright field and YFP images were exported from LasX (Leica Microsystems) as TIFF files and transferred into a grayscale format. These images were used to calculate YFP signal intensity using FIJI software (Schindelin et al. 2012).

ROI was manually defined using bright field images to measure average signal intensity for each plant. ROI was then superposed onto their corresponding YFP images. Mean brightness was then measured for each frame.

II) Automated measurement with a single ROI

A single ROI was defined using bright field images taking into account plant movements to establish a fixed ROI measurement. YFP signal intensity quantification using a single ROI was analyzed using LasX software (Leica Microsystems) for images taken over the course of the experiment.

III) Tracking plant movements using our automated measurement technique

Images from Texas Red and YFP filters were separated using a Red-Blue-Green spectrum to generate automated signal intensity measurements. Red channel images were separated from Texas Red filtered images; green channel images were separated from YFP filtered images; other channels were not used.

The *VSP1* expression of freely moving plants were traced automatically by overlaying the Texas Red images (in which plant shapes are visible) and YFP images (which represent *VSP1* expression). YFP signal intensities within Texas Red regions were calculated with the assistance of the "Analysis" function of the LasX software (Leica Microsystems).

An overview of the workflow is outlined in Figure 3a. Details for setting up and executing the automatic measurements are provided in Supplementary Material 1. Macro file to facilitate this execution is also provided in Supplementary File.

Results and discussion

To observe fluorescent signals in freely moving plants, we placed Arabidopsis plantlets under a stereomicroscope. As we expected to observe fluorescent signals in Arabidopsis shoots, we recorded time-lapsed images from an overhead view. After a 12-hour observation period, we found that the plant leaves exhibited significant movement (Figure 1a).

To detect fluorescent signals from reporter gene activity in freely moving Arabidopsis leaves, we recorded images over a 24-hour period. We used a three-week old transgenic Arabidopsis plants that contained a transgene consisting of jasmonic acid (JA) responsive promoter *VEGETATIVE STORAGE PROTEIN1* (*VSP1*) fused with *YELLOW FLUORESCENT PROTEIN (YFP)* (Betsuyaku et al. 2018). We treated the plants with methyl dihydrojasmonate and recorded YFP signal emissions after the treatment.

A clear YFP signal induction within the moving plants was detected (Figure 1b). To quantify signal intensity, we selected a ROI to cover all plant movement. The result of this method was consistent with our visual observations (Figure 1b, 1c).

Analyzing signal intensities using a single ROI required a large amount of space because the plant leaves moved freely. This created a large amount of background space (Figure 2a). We manually traced sample shapes using bright field images to measure fluorescent signals while minimizing background space. Rather than using
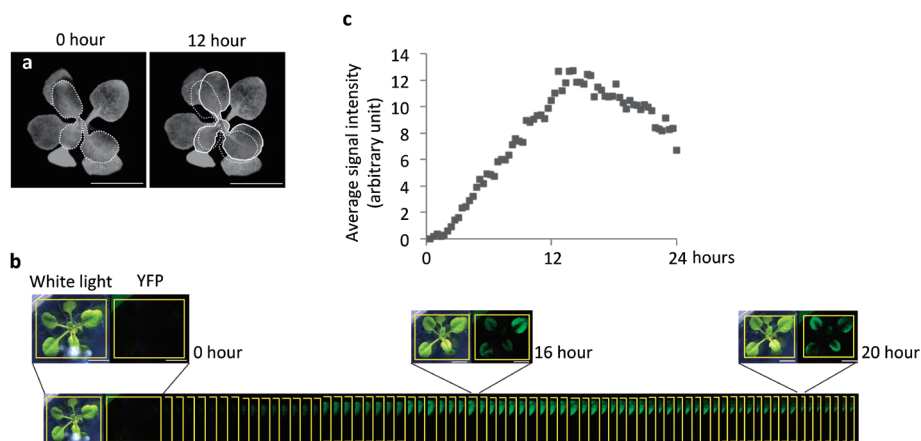
Figure 1. Plant movement. (a) Movement of the Arabidopsis leaves over 12 h of time-lapse imaging from an overhead view. Dotted lines outline the contour of the upper three leaves at 0 h. The solid line denotes the position of the same leaves after a 12-hour interval. Scale Bar: 5 mm (b) Schematic view of YFP measurement in which a single ROI was selected to cover plant movements. The yellow line indicates ROI. (Top) Bright field (Left) and YFP (Right) images from 0, 16, and 20 h. (Bottom) Representative view of 70 frames using a single ROI for 24 h. (c) Measurement of YFP intensity during 24 h in 70 frames using ROI from b. Each square indicates a single frame.
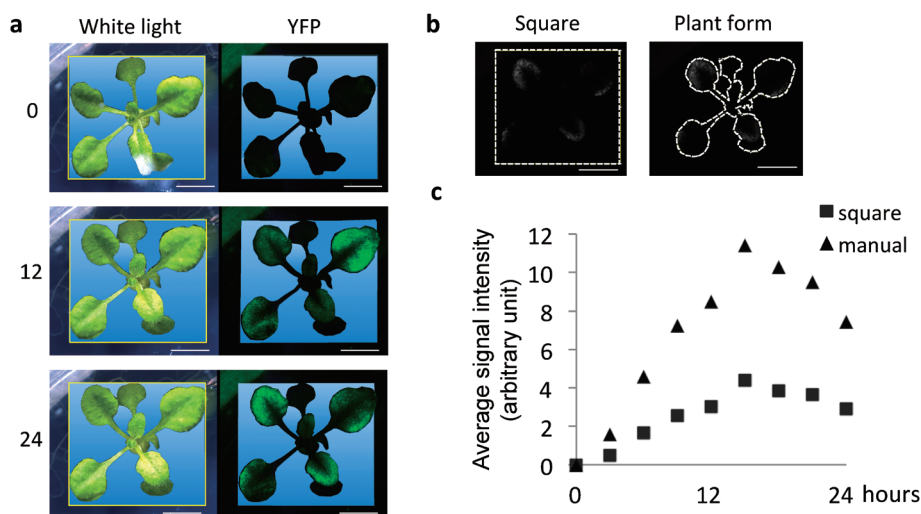

Figure 2. Empty space found within a single ROI. (a) Left panel shows bright field images. Right panel shows YFP images. Top, 0 h; Middle, 12 h; Bottom, 24 h from the start of the experiment. Blue area indicates regions within the ROI not occupied by plants. Yellow line indicates ROI. Scale Bar: 5 mm (b) Examples of fixed ROI (square) and manually defined ROI following plant shapes using bright field images for each frame. (c) Average values of brightness were measured using FIJI software with a fixed ROI (square) and manually defined ROIs (triangle).

a fixed square ROI (Figure 2b left), the shape of the plant was registered as the ROI (Figure 2b right).

Since it was impractical to manually define ROI for each of the 70 frames recorded, we registered ROI for every 8th frame. Using an identical data set, we then measured the signal intensity for each registered frame. The results of our manual shape tracking analysis exhibited a similar pattern to that of a fixed ROI (Figure 2c). Defining ROI by automatically tracing plant shapes resulted in higher sensitivity in measuring signal intensities than fixed square ROIs. This can be explained by the reduction of noise due to the minimization of background when manually tracing plant shapes. Because of the limited number of frames analyzed, temporal resolution was also reduced over time. We were able to

overcome the challenges of manual definition using the automated technique we developed for tracking ROI. Figure 3a contains a detailed outline of the procedure.

We used chlorophyll autofluorescence as a proxy for plant shape because the plants were recorded from an overhead view. We separated autofluorescent signals from chlorophyll to correspond with bright field images. To accomplish this, we simultaneously recorded bright field images alongside Texas Red and YFP images. It is necessary to acquire YFP and autofluorescence in separate channel to retain specificity of each signal, which is essential for precision of the measurements. We then converted the data to grayscale (Figure 3b, 3c, 3g). The threshold of grayscale images from the Texas Red filter was adjusted to represent Texas Red filter and bright field
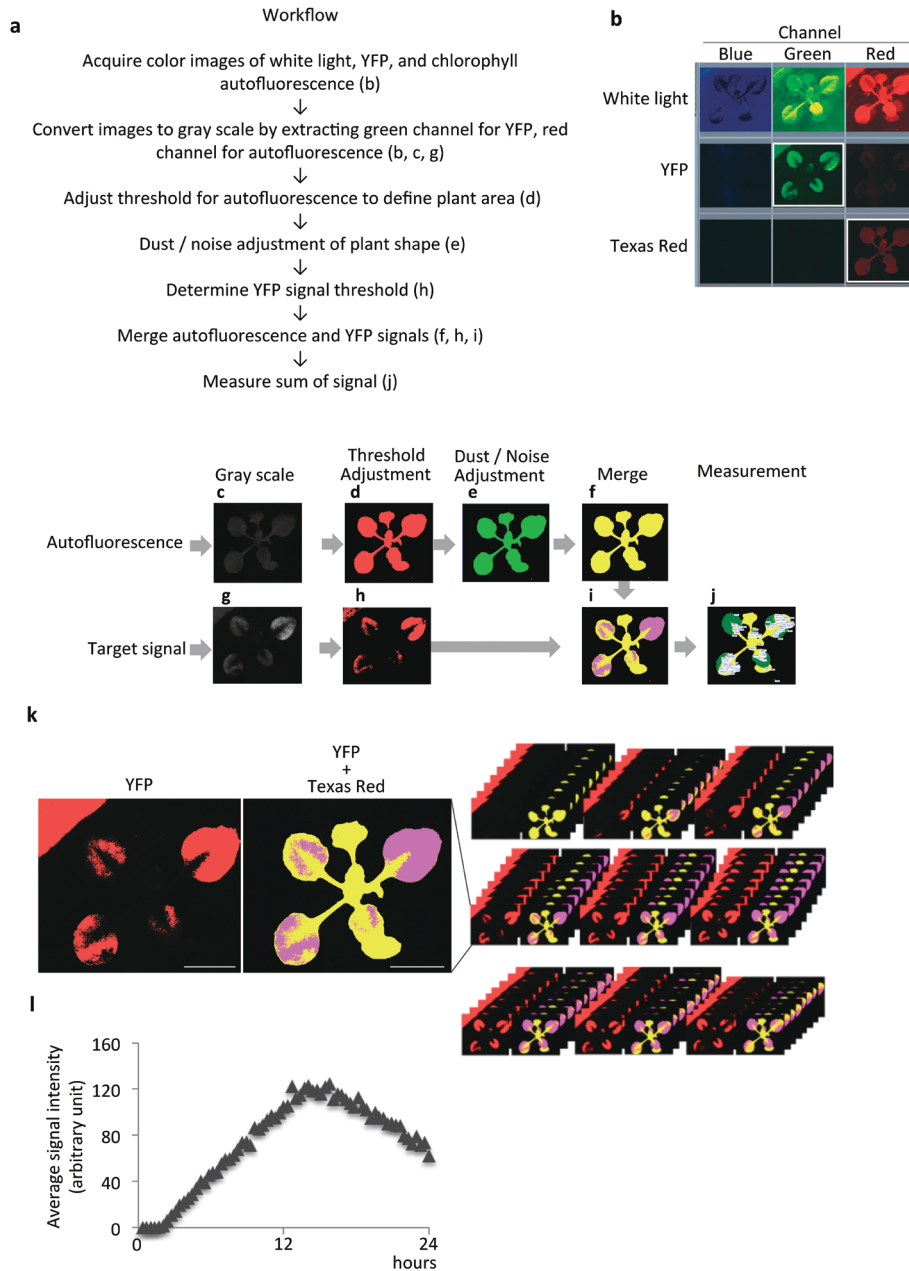
Figure 3.   Automated measurement of specific signals in moving Arabidopsis leaves. (a) Overview of the workflow. (b) Converting color YFP and Texas Red filtered images into grayscale images. White squares indicate selected channels. (c–j) Outline of step-by-step process with intermediate images to automate the tracing of plant movement. (k) Example of our method for automatic measurement over 70 frames. (Left panel) The left picture shows an image from the YFP filter that includes background signals. The right picture shows YFP signals that are present in both YFP and Texas Red filtered images. Magenta, both YFP and Texas Red are present; Yellow, Texas Red filtered images; Red; YFP filtered images. (Right panel) A representative illustration for 70 frames. (l) Measurement of YFP intensity during 24 h in 70 frames using our automated method. Triangles indicate mean value for YFP intensity for each frame.

images (Figure 3d). This procedure was followed by fine tuning the plant shape contour by using noise adjustment (Figure 3e). The threshold of grayscale images from the YFP filter were also adjusted (Figure 3g, 3h). These images, including background signals, were superposed onto their corresponding Texas Red filtered images to create merged images (Figure 3f, 3h, 3i). Only the YFP signals present within the boundaries of the plant shape were registered as target protein-specific signals. Background noise was

automatically excluded (Figure 3i, 3k).

In our analysis, we found that fixed ROI and shape recognition demonstrated consistent results (Figures 1c, 3l, Supplementary Material 2). Supplementary Material 3 exhibits an independent measurement of fluorescent signals to help validate our technique.

Situations where our technique is not directly applicable include examining specimens that do not emit chlorophyll autofluorescence like plant roots. In these

cases, signals from fluorescent proteins that are expressed utilizing constitutive promoters, for example 35S, can be used in place of autofluorescence (Benfey and Chua 1990; Curtis and Grossniklaus 2003; Larrieu et al. 2015; Rellán-Álvarez et al. 2015). Additionally, fluorescent proteins whose expression is regulated by organ tissue and cell-specific promoters enable signal tracking at targeted locations (An et al. 1996; Grefen et al. 2010; Marquès-Bueno et al. 2016). Fluorescent signals from transgenic constructs, however, are usually weaker than autofluorescence from chlorophyll. The use of transgenic constructs will also likely require longer exposure to the excitation light which stresses plants.

Conclusion

We developed automated shape recognition and tracking methodology that utilizes chlorophyll autofluorescence as a constitutive marker. This helped to: 1) enable objective evaluation of specific fluorescent signals during time-lapse experiments via automation; 2) minimize stress caused by immobilizing plants; 3) limit exposure to UV light to minimize plant stress; 4) measure the signal intensity of freely moving plants without employing specialized software; 5) trace plant movement and shape recognition without introducing a constitutive fluorescent marker gene.

References

An YQ, McDowell JM, Huang S, McKinney EC, Chambliss S, Meagher RB (1996) Strong, constitutive expression of the *Arabidopsis* ACT2/ACT8 actin subclass in vegetative tissues. *Plant J* 10: 107–121

Benfey PN, Chua NH (1990) The cauliflower mosaic virus 35S promoter: Combinatorial regulation of transcription in plants. *Science* 250: 959–966

Betsuyaku S, Katou S, Takebayashi Y, Sakakibara H, Nomura N, Fukuda H (2018) Salicylic acid and jasmonic acid pathways are activated in spatially different domains around the infection site during effector-triggered immunity in *Arabidopsis thaliana*. *Plant Cell Physiol* 59: 439–444

Bodnaryk R, Yoshihara T (1995) Structure–activity relationships of cyclopentane analogs of jasmonic acid for induced responses of canola seedlings, *Brassica napus* L. *J Chem Ecol* 21: 1735–1743

Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. *Plant Physiol* 133: 462–469

Grefen C, Donald N, Hashimoto K, Kudla J, Schumacher K, Blatt MR (2010) A ubiquitin-10 promoter-based vector set for fluorescent protein tagging facilitates temporal stability and native protein distribution in transient and stable expression studies. *Plant J* 64: 355–365

Grossmann G, Krebs M, Maizel A, Stahl Y, Vermeer JEM, Ott T (2018) Green light for quantitative live-cell imaging in plants. *J Cell Sci* 131: jcs209270

Huth J, Buchholz M, Kraus JM, Schmucker M, von Wichert G, Krndija D, Seufferlein T, Gress TM, Kestler HA (2010) Significantly improved precision of cell migration analysis in time-lapse video microscopy through use of a fully automated tracking system. *BMC Cell Biol* 11: 24–36

Jones AM, Danielson JA, Manojkumar SN, Lanquar V, Grossmann G, Frommer WB (2014) Abscisic acid dynamics in roots detected with genetically encoded FRET sensors. *Elife* 3: e01741

Khushi M, Napier CE, Smyth CM, Reddel RR, Arthur JW (2017) MatCol: A tool to measure fluorescence signal colocalisation in biological systems. *Sci Rep* 7: 8879–8887

Kinoshita N, Betsuyaku S (2018) The effects of Lepidopteran oral secretion on plant wounds: A case study on the interaction between *Spodoptera litura* and *Arabidopsis thaliana*. *Plant Biotechnol* 35: 237–242

Kishimoto K, Matsui K, Ozawa R, Takabayashi J (2006) Analysis of defensive responses activated by volatile allo-ocimene treatment in *Arabidopsis thaliana*. *Phytochemistry* 67: 1520–1529

Komis G, Šamajová O, Ovečka M, Šamaj J (2015) Super-resolution microscopy in plant cell imaging. *Trends Plant Sci* 20: 834–843

Larrieu A, Champion A, Legrand J, Lavenus J, Mast D, Brunoud G, Oh J, Guyomarc'h S, Pizot M, Farmer EE, et al. (2015) A fluorescent hormone biosensor reveals the dynamics of jasmonate signalling in plants. *Nat Commun* 6: 6043

Marquès-Bueno MDM, Morao AK, Cayrel A, Platre MP, Barberon M, Caillieux E, Colot V, Jaillais Y, Roudier F, Vert G (2016) A versatile multisite gateway-compatible promoter and transgenic line collection for cell type-specific functional genomics in *Arabidopsis*. *Plant J* 85: 320–333

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15: 473–497

Rellán-Álvarez R, Lobet G, Lindner H, Pradier PL, Sebastian J, Yee MC, Geng Y, Trontin C, LaRue T, Schrager-Lavelle A, et al. (2015) GLO-Roots: An imaging platform enabling multidimensional characterization of soil-grown root systems. *eLife* 4: e07597

Rizza A, Walia A, Lanquar V, Frommer WB, Jones AM (2017) In vivo gibberellin gradients visualized in rapidly elongating tissues. *Nat Plants* 3: 803–813

Rohloff J, Bones AM (2005) Volatile profiling of *Arabidopsis thaliana*: Putative olfactory compounds in plant communication. *Phytochemistry* 66: 1941–1955

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, et al. (2012) Fiji: An open-source platform for biological-image analysis. *Nat Methods* 9: 676–682

Waadt R, Hitomi K, Nishimura N, Hitomi C, Adams SR, Getzoff ED, Schroeder JI (2014) FRET-based reporters for the direct visualization of abscisic acid concentration changes and distribution in Arabidopsis. *Elife* 3: e01739