Automating measurements of fluorescent signals in freely moving plant leaf specimens

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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.

Abbreviations: Arabidopsis, Arabidopsis thaliana; JA, jasmonic acid; VSP1, VEGETATIVE STORAGE PROTEIN1; YFP, YELLOW FLUORESCENT PROTEIN.

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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
1) 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...
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.
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; Greff 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.

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