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# Use of Image Analysis Software as a Tool to Visualize Non-Radioactive Signals in Plant *in Situ* Analysis

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**Abstract.** *In situ* hybridization (*ish*) allows the visualization of gene expression in tissues at high microscopic resolution. Interference by plant tissue pigments generally confers higher sensitivity to radioactive *ish*, relative to non-radioactive *ish* using hapten labeled probes. The increased resolution is partially due to image acquisition methods in radioactive *ish* experiments. However, radioactive *ish* has many drawbacks including short probe life, safety concerns associated with the use of radioactive materials, and slow development of signal. In this report, we show how commercially available image analysis software can be used to extract data from non-radioactive *ish* images to gain a substantial increase in resolution. We provide a comparison between detecting a probe (*CELLULOSE SYNTHASE*) that is expected to produce a consistent, detectable signal in all growing tissues with detection of a probe (*LEAFY*) that is expected to produce a signal only in specific tissues.

*Key words:* Adobe Photoshop, image analysis, ImageJ, *in situ* hybridization, non-radioactive hybridization, plants, *Theobroma cacao*

**Abbreviations:** *ish*, *in situ* hybridization; NBT/BCIP, nitrotetrazolium blue chloride / 5-bromo-4-chloro-3-indoxyl phosphate.

## Introduction

*In situ* hybridization (*ish*) allows the visualization of gene expression using high magnification microscopic resolution. *Ish* is a technique whereby tissue is fixed, embedded in paraffin, sectioned, and adhered to a glass slide. Labeled DNA or RNA fragments of specific genes of interest are used as probes and are hybridized to the tissue sections. Hybridization of the probe reveals the localization of RNA in the tissue section. The spatial resolution of this method far exceeds that of northern blotting or RT-PCR methods by allowing identification of expression down to a single cell layer, and in some cases has been used to localize mRNA at a sub-cellular level (Jackson, 1992; Scutt, 2001). This allows precise determination of tissue and time specific expression patterns common in regulatory genes.

Currently the most common methods of signal detection in *ish* are by either radioactive or by non-radioactive means. Radioactive *ish* often uses $^{35}$S radioisotope labeled probes, while non-radioactive *ish* uses hapten (often digoxygenin) labeled probes. $^{35}$S probes resolve, on average, one cell in diameter and are regarded as

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being the most sensitive of all the aforementioned detection methods (Wilkinson, 1998). Hybridization is detected by using a Kodak NTB2 emulsion, then dark-field microscopy to detect silver grains corresponding to where the probe has hybridized. Data is collected by overlaying the dark-field photograph with a corresponding bright-field photograph (Ruzin, 1999). However, $^{35}$S labeled probes do have some drawbacks including: short probe life (often probes must be used within a month), problems with disposal of radioactive materials, and safety concerns associated with the use of radioactive labels (Wilkinson, 1998). The major drawback of $^{35}$S labeled probes is long exposure times, which have been reported as being between 2 – 6 weeks depending on the probe and abundance of the target transcript (Zahn et al., 2005).

Hapten probes, although regarded as being less sensitive, do confer several advantages with regard to safety, disposal, and time, compared to $^{35}$S probes (Lahaye et al., 1996; Wilkinson, 1998). Digoxygenin labeled probes can be developed via antibody:NBT/BCIP detection (generally a blue precipitate) in 3–4 days, a clear advantage when compared with the 2–6 weeks required for detection of $^{35}$S labeled probes. In part, the lesser sensitivity of non-radioactive probes can be attributed to the method of data collection. In contrast to $^{35}$S labeled probes where two separate images are taken (one of the signal, and one of the background image) and overlaid, only a single image is recorded with non-radioactive probes that contains both the image and signal. Having no way to separate the two images can make it more difficult to discern signal from background when non-radioactive probes are used, especially if the observed tissue is highly pigmented.

In this article, we describe a method of enhancing the discrimination of the signal from background using the photo editing software Adobe Photoshop®. The signal is false-colored and superimposed on the original image, making the hybridization signal much more obvious, thus significantly improving the analysis of non-radioactive *ish*.

## Materials and Methods

Six to eight day old flower buds from *Theobroma cacao* L. (clone PSU-"SCA6") were used (Swanson, 2005). *Ish* analysis was carried out by combining the radioactive protocol of Zahn et al. (2005) with the protocol of Lin (2000). The Lin (2000) protocol is a modification of the protocols of Hake, Irish and Meyerowitz (Meyerowitz, 1987; Jackson, 1992). *T. cacao* ESTs of the genes *CELLULOSE SYNTHASE* (DQ149726) and *LEAFY* (DQ149725) were selected based on their expected contrast in gene expression. In general, it was expected that *CELLULOSE SYNTHASE* would be expressed throughout the growing tissues, while *LEAFY* expression would be restricted to the floral and leaf meristematic tissues (Weigel et al., 1992; Amor et al., 1995).

The resulting *ish* slides were viewed on a Nikon Optispot microscope (Nikon, Garden City, NY), and sections showing signal were photographed using a Spot RT Slider digital camera (Diagnostic Instruments, Sterling Heights, MI) (see Figures 1A and 1D). The images were subjected to further analysis using Adobe Photoshop® CS (Adobe®, San Jose, CA) and ImageJ v. 1.33u (NIH, USA) as described below in Results. The NIH ImageJ software is freely available for

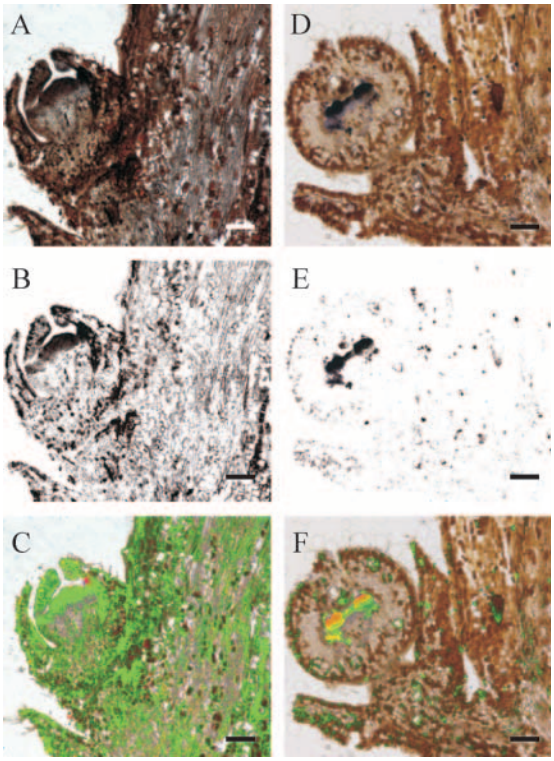


*Figure 1. In situ* hybridization of *CELLULOSE SYNTHASE* (A-C), and *LEAFY* (D-E) genes to *T. cacao* 6–8 day old flower buds. (A and D) Raw images of NBT/BCIP (blue precipitate) stained *ish* hybridizations. (B and E) Extraction of the NBT/BCIP precipitate color (0-104 (Y), 120-144 (U), 113-142 (V)), from the raw image using Adobe Photoshop® (C and F). The superimposition of the image of the recolored signal (changed to green) onto the original raw image, showing hybridization signal that was not apparent in the raw images. Scale bar is 100 μM.

download at the ImageJ web site http://rsb.info.nih.gov/ij/ along with documentation, manuals, and other resources.

## Results and Discussion

Using Adobe Photoshop® CS the "blue hues" associated with the NBT/BCIP precipitate were selected from the images in Figure 1, panels A and D (using the select > color range tool) (Figures 1B and 1E) and replaced (using image > adjustments > replace color tool) with colors that allowed the probe to be more easily detected (Figures 1C and 1F). The resulting color selection could be saved as a "color mask" file so multiple images from the same *ish* experiment could be easily analyzed. It is important to note that since the blue hues could vary from experiment to experiment (due to variations in development time, and quality of the NBT/NCIP dye); color selection was done once per *ish* experiment. The resulting Adobe Photoshop® color range file was used to apply the filter identically on all images of the same experiment, including control sense strand hybridizations.

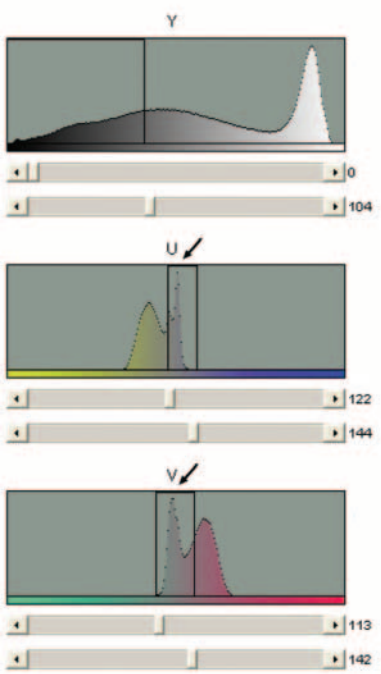


*Figure 2.* Output of analysis of the YUV color space of a typical *ish* image using ImageJ v. 1.33u with the Color Threshold plug-in. Arrows above U and V graphs indicate peaks in color intensity associated with the NBT/BCIP blue precipitate. Boxes indicate the regions used to extract the NBT/BCIP signal, with the corresponding sliders below showing the bright values selected. The x-axis on each graph represents a 0-255 unit range in arbitrary brightness values, while the y-axis shows the number of pixels at each brightness value.

The selected blue hues depicted in Figure 1 were analyzed using the Threshold Color plug-in in the image analysis program ImageJ v. 1.33u. The ImageJ program found that the blue hues were represented by the following YUV color space ranges: 0-104 (Y), 120-144 (U), and 113-142 (V) (Figure 2). It was noted that especially in the U and V channels specific separate peaks could be identified corresponding to the NBT/BCIP precipitate (Figure 2, arrows). It is also possible using ImageJ and the Threshold Color plug-in to produce images identical to those created by Adobe Photoshop® (Figures 1B and 1E).

The unprocessed images obtained from *ish* of *CELLULOSE SYNTHASE* and *LEAFY* on *T. cacao* tissue (Figures 1A and 1D), illustrate how difficult it can be to identify differences in gene expression between two genes from such standard non-radioactive *ish* images. However using the image enhancement capability of Adobe Photoshop® (or ImageJ v. 1.33u with the color threshold plug-in), it was possible to extract and enhance the signal from the images. The results in Figures 1B and 1E, demonstrate that the natural brown hues of the tissue no longer obscure the NBT/BCIP precipitate after color-enhancement. Figure 1B also confirms that *CELLULOSE SYNTHASE* is expressed throughout the growing tissues. In contrast, the *LEAFY* gene is expressed exclusively in the meristematic tissues

(Figure 1E). Finally, it is possible to combine the background image and signal images for cellular localization of signal (Figures 1C and 1F).

These results show the applicability of Adobe Photoshop® to overcome the major drawback of tissue pigmentation for non-radioactive *ish* in plants, by extracting and superimposing images specific to the signal from the hybridized probe. Our data provides evidence that non-radioactive probes, when used in combination with Adobe Photoshop® (or ImageJ), can provide high-resolution detection of gene expression in substantially less time and without the safety concerns inherent in the use of radioactivity.

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