1176
doi:10.1017/S1431927616006723

Microsc. Microanal. 22 (Suppl 3), 2016

Image Analysis of Transient Expression in Bombarded Soybean (*Glycine max*) Immature Embryos.

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Particle bombardment of cells traditionally results in low frequencies of regenerated transgenic plants, which could be due to low (1-2%) cell viability in particle containing cells (Hunold, Bronner, & Hahne, 1994). Several methods for reducing damage to cells during bombardment have been developed, including the use of osmotic treatments (Vain, McMullen, & Finer, 1993) and the reduction of gold particle size (Randolph-Anderson, et al., 1995). It was determined that reducing the total number of gold particles per shot at the target tissue might reduce the damage received by each cell, thereby improving cell survival and regeneration. Immature embryos of soybean were bombarded using five different concentrations of gold particles to determine the effects of gold particles as measured by transient expression. Image analysis of bombarded immature embryos was performed using ImageJ to determine the number and location of foci with the bombardment target area and this information was used to optimize concentration of gold density for particle bombardment.

Quantification of transiently expressing bombarded cells was performed using ImageJ Fiji (Schindelin, et al., 2012) image analysis software. Twenty four hours post bombardment, six to ten images of each bombarded plate were captured and then stitched together using MosaicJ plugin (Thevenaz & Unser, 2007) (Figure 1 A). The image is separated into three (red, green, and blue) 8 bit images (Figure 1 B and C), and green and blue are discarded. Immature embryos were bombarded with a turbo RFP containing plasmid so only the red channel is used for the analysis. A threshold filter is applied to the image so that only the target material is shown. Using the ImageJ wand tracing tool, the target area can be measured for total target area (Figure 1 D) and the find maxima tool total produces foci count and positional information (Figure 1 E). Two experiments were conducted for testing the effect of gold density. In the first experiment, three gold concentrations (1000, 500, and 250 µg gold per shot) were tested with about 26 plates, each containing 10 immature embryos. In the second experiment, five gold concentrations (1000, 500, 250, 125, and 62.5 µg of gold per shot) were tested with about 12 plates per treatment. Control images were analyzed in a similar manner to produce an expected background per unit area foci count using the same noise tolerance used in the find maxima tool for the experimental analysis. The background foci per unit area was multiplied by the total area in experimental images to generate the number of expected background (false) foci which was then subtracted from the foci count of the experimental image (background adjusted foci count).

With a decrease in gold density, an increase in average foci counts was observed. In the first experiment, 250 and 500 µg of gold per shot were found to be statistical higher than 1000 µg of gold per shot (Table 1). In the second experiment, the average foci counts were highest with 125 and 62.5 µg per shot while 250 and 1000 µg were the next highest and 500 µg had the lowest foci counts (Table 1). In both the first round and second round experiments, spatial location of foci was collected and plotted to produce a foci density map. Individual foci maps showed a variety of expression patterns in the bombardment target area. This high degree of variability limited attempts to determine patterns in plotted bombardment densities, aside from location of immature embryos within the bombardment target area. Combining several replicates of the same treatment in the same density map produced a pattern of foci density that was observable (Figure 2). In the case of high gold particle density (1000 µg gold per shot), immature embryos positioned at the center of the target area, in aggregate, had less foci then those positioned on the periphery. Though the difference between central and periphery explant transient expression was not found to be statistically significant, the average foci counts at the center of the bombardment improved, as gold density reduced. We believe this difference is linked to multiple particles bombarded to the same cells under high gold density conditions leading to high cell mortality. We believe this method of image analysis has broad applications in the further optimization of bombardment parameters as well as foreign gene expression studies.

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Table 1 Average background adjusted foci counts for experiment 1 and 2

Treatment (µg gold/shot)	Experiment 1		Experiment 2	
	Embryos	Avg. Foci*	Embryos	Avg. Foci*
62.5	130	1555.4 a		
125	110	1721.4 a		
250	121	1401.4 ab	260	3341 a
500	120	965.7 c	260	3152.5 a
1000	120	1043.1 bc	270	2561.4 b

*Treatments not connected by the same letter are significantly different (Tukey's HSD p<.0001)

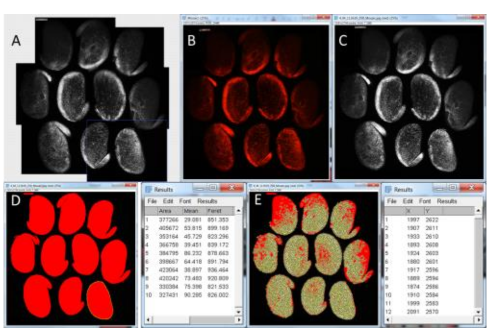

Figure 1. Bombardment Image processing

A) Stitching with MosaicJ tool, B) Stitched image, C) Red channel, D) Target area outlined and measurement of bounded area, E) foci count/ position (find maxima)

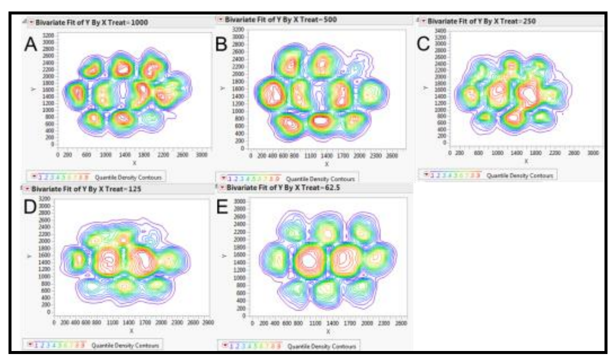

Figure 2. Density map of bombardments

A) Treatments 1000, B) 500, C) 250, D) 125, and E) 62.5 µg gold/ shot.

https://doi.org/10.1017/S1431927616006723 Published online by Cambridge University Press