

— Note on Methodology —

QUANTIFYING POLLEN DEPOSITION WITH MACRO PHOTOGRAPHY AND ‘STIGMAGRAPHS’

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Abstract—The pollen deposited during a single visit by a flower visitor (“single-visit deposition”; SVD) is often measured by removing the stigma from the flower and counting the pollen grains deposited under a microscope. This process precludes study of any subsequent interactions between the flower and later visitors (such as pollen removal from the stigma). Furthermore, if the stigma is excised too soon after the pollinator visit, the flower may be rendered infertile, such that any analyses of fruit or seed yield in relation to pollen deposition must be done indirectly. Here, a method of pollen deposition measurement was developed using macro photography and the open-source image-analysis software program ImageJ/Fiji. Using colour segmentation options within the program, the pollen grains can be distinguished from the background stigmatic surface, and the percentage of stigma coverage can be calculated. This pollen deposition measurement method leaves the sampled flower in the field to develop into fruit, allowing any subsequent yield or quality analyses to be conducted directly.

Keywords: Floral stigmas, macro photography, pollen count, pollen deposition, pollen load, stigmagraph

INTRODUCTION

Experiments in pollination ecology often require a measure of the amount of pollen deposited onto a flower’s stigma. A measure of pollen deposition is required in order to compare pollen-transfer and pollen-deposition efficiencies among different species of flower visitors (Schemske & Horvitz 1984; Motten et al. 1981; Javorek et al. 2002; Adler & Irwin 2006; Garibaldi et al. 2013) or simply to distinguish between flower visitors and true pollinators (King et al. 2013). Pollen deposition measurements are also used when assessing the influence of pollen quantity on fruit and seed set in wild and agricultural plants (Falque et al. 1995; Waites & Ågren 2004; Ne’eman et al. 2010).

It is difficult to measure the pollen load on a flower’s stigma due to the small size of most stigmas, the vast number of pollen grains that can be deposited, and the potential to dislodge pollen during the measurement process. Further, because of the three-dimensional nature of many stigmas, pollen can be deposited in several focal planes, making the grains difficult to count (Flanagan et al. 2009). A widely used technique to determine the pollen load on floral stigmas is the stigma squash method (Kearns & Inouye 1993, Dafni et al. 2005, section 3.10). The stigma squash relies on a visual count of the pollen grains on a flower’s stigma under a microscope. Counting pollen on a squashed stigma can be a time-consuming process, and subsampling is often required when thousands of pollen grains have been deposited on a

single stigma. A stigma squash also requires the flower’s stigma to be removed from the field. If a flower’s stigma is removed too soon, i.e. prior to pollen-tube growth and ovule fertilization, analyses of seed or fruit production by the same flower are not possible (Faegri & Van der Pijl 1979; Kearns & Inouye 1993). Lastly, a flower is usually visited by multiple pollinators, and pollen counts determined through a stigma squash cannot determine each individual species’ contribution to the total stigmatic pollen load.

The purpose of the present study was to develop a pollen-deposition measurement technique that could be used to accurately quantify pollen deposition on stigmas, partition the stigmatic pollen load by pollen vector, and allow the sampled flower to remain in the field to develop fruit and seed. These particular criteria were chosen as they are important for research in pollination ecology and for studies relating pollination to fruit and seed development. Image analysis methods have successfully been used to count the number of pollen grains within flower anthers and on stigmas in a laboratory setting (Costa & Yang 2009; MacIvor et al. 2013), but had not been tested on flower stigmas that are to remain in the field. The ‘stigmagraph’ method of pollen deposition measurement developed here uses macro photography and image-analysis software to measure pollen deposition *in situ*. A stigmagraph is a photograph of a flower’s stigma, taken in the field and subsequently analyzed to determine the pollen count. Two species of daffodil (*Narcissus pseudonarcissus* and *Narcissus nanus*) were used to test and compare the stigmagraph technique to the standard stigma-squash technique. Pumpkin (*Cucurbita pepo* L.) flowers were used to determine if individual pollen grains on larger stigmas could be counted

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using the stigma graph method. Pollen deposition was also quantified on strawberry stigmas (*Fragaria* × *ananassa*) using the stigma graph technique and compared to the seed set of the developed fruit.

MATERIALS AND METHODS

Stigma photography

First, a photo is taken of the virgin stigma of the sample flower before any flower visits have taken place. This is done to determine the baseline amount of pollen (if any) deposited on the stigma prior to any flower visits, and to accurately determine the amount of pollen deposited by each visitor thereafter. A photograph (hereafter a 'stigma graph') of the sample flower's stigma(s) is then taken after each flower visit. Each stigma graph needs to be taken at the same angle, lighting, and camera aperture setting to ensure consistency for image analysis. For a stigma graph to be adequate for image analysis, it must be taken with a camera fitted with a dedicated, macro lens (at least 1:1 magnification). The type of lens used will depend on the size of the stigmas of the sample flower and the working distance required. A standard macro lens, such as a Canon EF 100 mm macro lens, is adequate for flowers with a pistil surface of greater than 2 mm in diameter. Macro photography inherently involves working with a very shallow depth-of-field (DOF). To achieve the largest depth-of-field possible, the photos should be taken with a small aperture opening ($f/16$). Photographs taken further away from the flower will increase DOF but sacrifice image detail. This is undesirable for the subsequent photo analysis and detection of pollen grains. If the stigmatic surface is not flat, as is often the case, it is best to capture a series of images of the stigma surfaces at increasing focal depths. Focus stacking, or focal plane merging, can then be performed on the images at the processing stage (Johnson 2008).

Once the photos of the stigma(s) are captured in the field, the photos are processed through ImageJ/Fiji to determine the amount of pollen deposited on the stigma. ImageJ is a free, Java-based image-processing program and Fiji is an open-source image-processing package which operates within the ImageJ software environment (Schneider et al. 2012; Schindelin et al. 2012). For flowers with simple morphology, such as the single-pistiled daffodil (*Narcissus* spp.), pollen grains can be distinguished from the background stigmatic surface using the segmentation options within ImageJ, namely colour thresholding. Two *Narcissus* spp. were used here to test the stigma graph method with two different-sized pollen grains, and because they were readily available in the field.

Photographs of several pumpkin (*Cucurbita pepo* L.) stigmas were taken after a series of flower visits to determine if the number of pollen grains deposited on larger stigmas could be counted in a set of focus-stacked photos using the Fiji Cell Counter plugin. By adjusting the camera angles, the full extent of the stigmatic surface can be captured, but this requires time and manipulation of floral structures. As such, the pumpkin stigmas were only photographed from the top

down; the underside of the stigmas was not captured in the stigma graphs.

For more complex flowers, such as the multi-pistiled strawberry (*Fragaria* spp.), the Trainable Weka Segmentation application (Hall et al. 2009) is more suitable to determine the total stigmatic pollen load. Alternatively, on a flower with multiple pistils, the number of stigmas carrying pollen can be counted from the photographs using Cell Counter. This is the simplest option for a researcher requiring a measure of the number of stigmas having received pollen. Strawberry was chosen as a test flower because of the multi-pistiled morphology and the ability to assess successful pollination from the outside of the fruit. The three photo-processing options used to calculate the proportion of the stigmatic surface covered by pollen—ImageJ Colour Thresholding, the Trainable Weka Segmentation and manual counting with Fiji's Cell Counter—are described in detail below.

Measuring pollen deposition with ImageJ Colour Thresholding

The proportion of the stigmatic surface covered by pollen can be calculated using ImageJ Colour Thresholding (ICT) as follows:

1. Open the image of the virgin stigma in Fiji. Convert the image to a RGB colour photograph under 'Image' in the *Type* menu.
 2. To remove the background, use the 'Adjust colour threshold' option under the *Image>Adjust* menu. Select the 'Dark background' option to fill the stigmatic surface in black (Fig. 1A). Click the 'Select' button, and close the *Colour Threshold Window*. Then select 'Clear outside' under the *Edit* menu. This will remove the background and leave only the stigmatic surface in the photo. Select 'Binary' under the *Process* menu and select 'Make binary'. This will create an image similar to Fig. 1A.
 3. Select 'Histogram' under the *Process* menu and then select 'List'. This will open a table of values. The first column of the table contains a list of colour values; the second column contains the area of each colour, in pixels. The area corresponding to colour-value 255 (black) is the area of the stigmatic surface.
- Note: To measure the pollen deposition in terms of proportion of stigmatic area, a scale is not needed. If a measurement of pollen deposited in mm² or cm² is desired, a scale can be set under the *Analyze* menu. Only measurements of the proportion of the stigma surface covered in pollen are described here.
4. Open the next image of the sample stigma, taken after the flower has received a visit. Repeat step 1, and use the 'Adjust colour threshold' option under the *Image>Adjust* menu and de-select the 'Dark background option'. Then click 'Select' and close the *Colour Threshold Window*. Then select 'Clear' (not 'Clear Outside') under the *Edit* menu.
 5. Open the *Colour Threshold Window*. Move the sliders on the 'Hue', 'Saturation', and 'Brightness' options

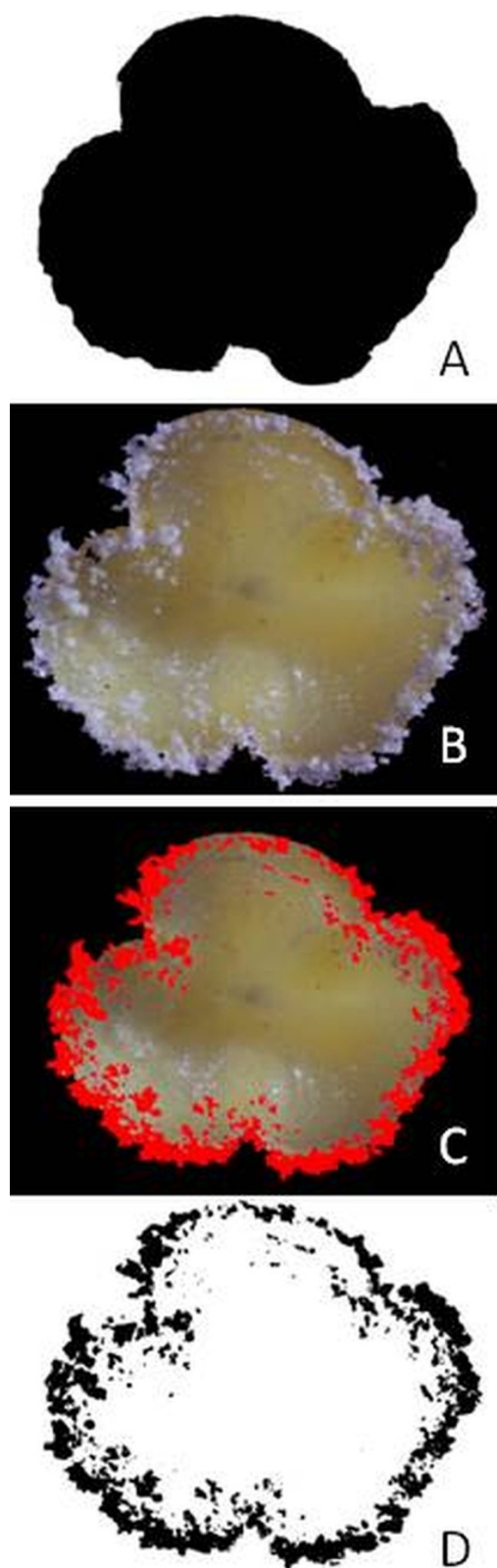


FIGURE 1. Quantifying the amount of pollen in the stigma photographs (stigmagraphs) with ImageJ Colour Thresholding (ICT) A) Binary photo of a virgin daffodil stigma (*Narcissus pseudonarcissus*). B) The same stigma with background removed. C) The segmented photograph, with pollen grains selected using colour thresholding. D) The binary photo of pollen deposition used to create a histogram and quantify the proportion of the stigmatic surface covered by pollen.

until only the pollen is highlighted in red. This effectively allows only those colours associated with pollen to pass and thus segments the photo into pollen and non-pollen areas (Fig. 1C).

6. Select 'Binary' under the *Process* menu and select 'Make binary'. This will create an image similar to Fig. 1D. Select 'Histogram' under the *Process* menu and then select 'List'. This will open a table of values. The value next to 255 (black) in the table is the area in pixels of the stigmatic surface covered by pollen.

7. Divide the number of black pixels obtained in Step 6 by the number of pixels obtained in Step 3 to obtain the proportion of the stigma covered by pollen.

Measuring pollen deposition with Trainable Weka Segmentation

For flowers of more complex morphology, the proportion of the stigmatic surface covered by pollen can be calculated using the Trainable Weka Segmentation (TWS) application, as follows:

1. TWS is a tool built into the Fiji version of ImageJ which can be accessed through the 'Segmentation' option in the *Plugins* menu.

2. Once in the TWS environment, zoom in on the stigmagraph(s) as much as possible. Use the *Freehand selection* tool to manually select areas of the photograph that contain pollen and pick 'Add to Class 1.' Once 2 or more areas clearly containing pollen are selected, select areas that do not contain pollen and add them to 'Class 2'. Then click *Train classifier*. This trains the application to define colours in Class 1 pixels as pollen, and Class 2 pixels as non-pollen (Fig. 2A).

3. Once the classifier training is finished (this can take a while, depending on computational power), select *Create result* in the left-hand menu. This will create an output similar to Fig. 2B.

4. Convert the image to binary (Fig. 2C) and obtain a histogram of colour values, as described in Step 6 of the ImageJ Colour Thresholding section (above). The amount of pollen in the photo is the number of black pixels indicated in the histogram table.

Measuring pollen deposition with Cell Counter

The Fiji Cell Counter can be used to count and track particles or objects in an image. Cell Counter can be used to count stigmas containing pollen as follows:

1. Select the *Cell Counter* plug-in under *Plugins* in the *Analyze* menu.

2. Initialize the photo by clicking the initialize button in the *Cell Counter* window.

3. Rename *Type 1* and *Type 2* to "Pollen" and "No Pollen", respectively (Fig. 3B).

4. Select *Type 1* and use the *Arrow* tool to click on the stigmas that contain pollen grains.

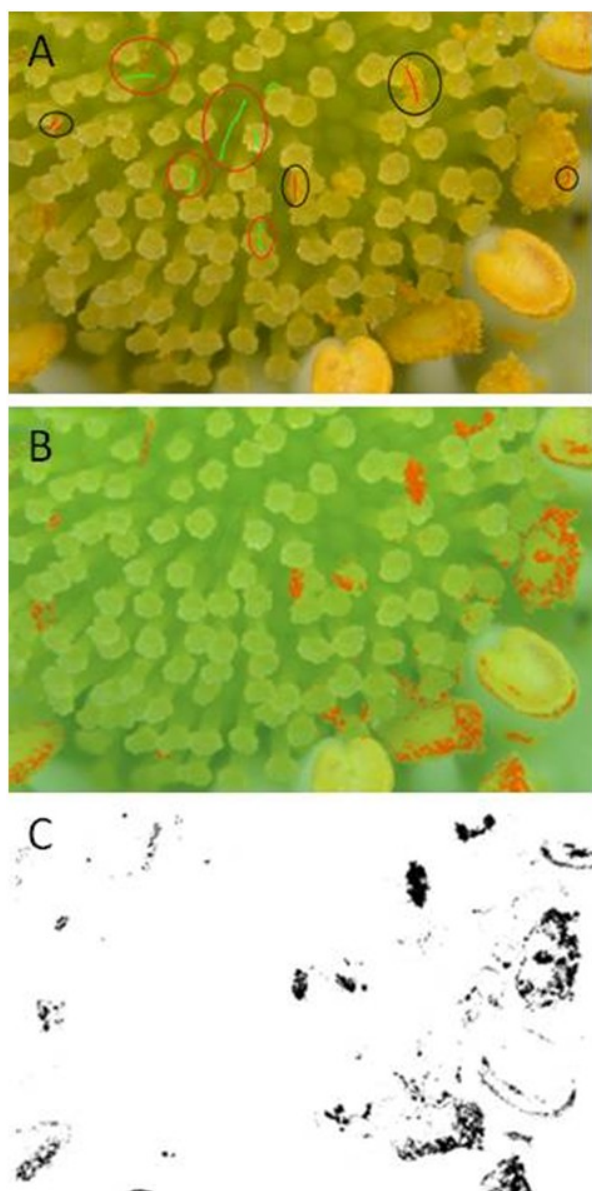


FIGURE 2. Section of macro photograph of strawberry (*Fragaria* × *ananassa*) stigmas and several anthers after processing through the Trainable Weka Segmentation (TWS) application in ImageJ. A) The TWS training window; red lines are the classification selections for pollen (circled in black), and green lines are the classification selections for non-pollen (circled in red). B) The output of the TWS. The orange areas indicate sections classified as pollen. C) The binary image used for the total pollen deposition calculation.

5. Select *Type 2* and use the *Arrow* tool to click on the stigmas that do not contain pollen grains.

6. Select *Results* to obtain a table containing the number of stigmas with and without pollen.

Comparing the stigmagraph method to the stigma-squash method

To test the ICT and the TWS measurements against the standard stigma-squash technique, 11 flowers of *Narcissus*

pseudonarcissus and 11 flowers of *Narcissus nanus* were hand-pollinated by lightly touching a dehiscing *Narcissus* anther to each stigma. The stigmas were then removed and photographed. In each photo, the proportion of the stigma covered in pollen was determined (in pixels) by both ICT and TWS. This proportional area of pollen on the stigma surfaces was then divided by the surface area of one *Narcissus* pollen grain to estimate the number of pollen grains on the stigma. The ICT method was performed twice on each stigma - once by an experienced stigmagrapher and once by an observer new to the stigmagraph method, in order to compare the processing time between users. The TWS measurement was only conducted once due to limited computational power. The time it took to process each stigmagraph with ICT was recorded for 12 stigmagraphs (6 *N. nanus* and 6 *N. pseudonarcissus*). The timing of the ICT method began when the photos were transferred to the computer and open in ImageJ, and ended when the user determined the number of pollen grains on the stigma.

Once photo processing was completed, the stigmas were squashed on microscope slides with fuchsin-stained gelatin. The pollen grains were counted on each stigma at 400 × magnification once by each observer, without sub-sampling. For 12 stigma-squash slides (6 *N. nanus* and 6 *N. pseudonarcissus*), the time required to complete the pollen counts on the stigma squash was recorded for each observer. The stigma-squash timing began once the stigma was mounted on the slide and ready to be counted.

Comparing stigmagraph pollen counts to seed set

In the spring of 2015, 120 commercial strawberry plants (*Fragaria* × *ananassa*, “Jewel” variety) were haphazardly selected at the McGill Horticultural Research Farm, Montreal, QC (45°24'39.6" N, 73°56'12.2" W) for a comparison of the number of stigmas containing pollen, as calculated using stigmagraphs, and seed set. A strawberry flower contains many carpels, each of which contains one ovary and one ovule. Once successful fertilization occurs, the ovaries of the strawberry flower develop into one-seeded achenes. Hereafter, seed set refers to the number of fertilized achenes per strawberry. The development of achenes is what stimulates the growth of the edible tissue of the receptacle (Nitsch 1950). Thus, the weight and shape of the berry is directly related to the number of achenes on a strawberry, which largely depends on the number of ovules successfully fertilized through pollination.

The selected strawberry plants were covered with cotton mesh at the bud stage and one primary flower of each plant was labelled. Once flowers were open, the mesh was removed to allow pollinators to visit and the flowers were photographed after each pollinator visit. To vary the quantity of pollen deposited, flowers were exposed to a varying number of pollinator visits (1–10); mesh was then replaced over the flowers. The strawberry variety used in this study was protogynous and the sampled flowers were not emasculated, so the flower stigmas were also photographed two days later to capture changes in the pollen load due to autogamous pollen deposition. After all photographs had been taken, the strawberry plants were kept covered until the stigmas began to turn brown and the petals had abscised.

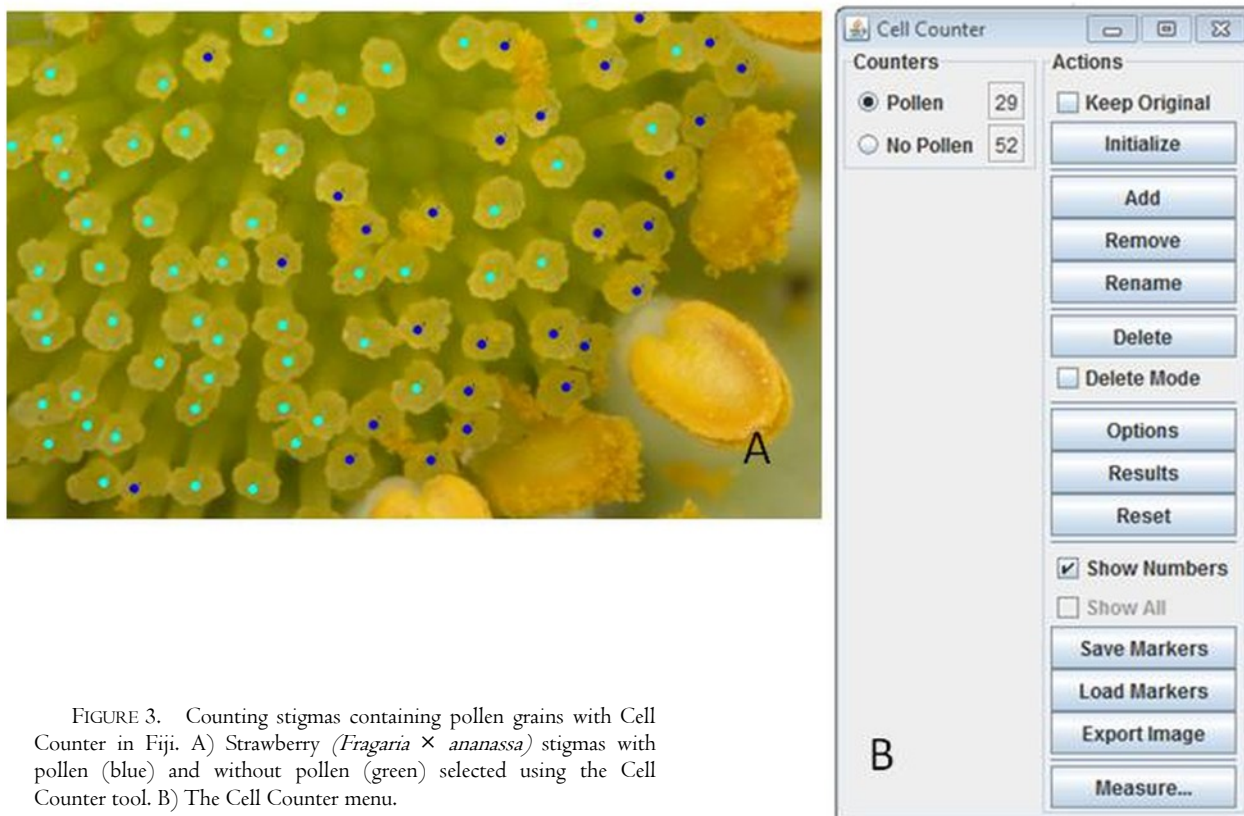


FIGURE 3. Counting stigmas containing pollen grains with Cell Counter in Fiji. A) Strawberry (*Fragaria* × *ananassa*) stigmas with pollen (blue) and without pollen (green) selected using the Cell Counter tool. B) The Cell Counter menu.

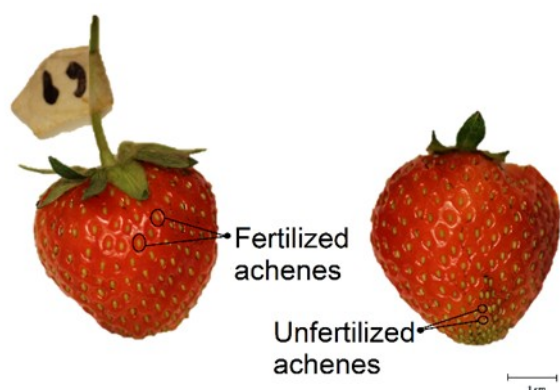


FIGURE 4: Fertilized and unfertilized achenes of a sampled strawberry. Fertilized achenes are those with a diameter > 0.4 mm and unfertilized achenes are those with diameter ≤ 0.4mm.

The total number of stigmas with pollen (including any autogamously deposited pollen) was calculated using the Fiji Cell Counter on the stigmagraphs taken at the end of receptivity, as described above. Once the fruit had developed, each strawberry was harvested and cut in half, and each side was photographed to avoid double-counting any achenes. Achene diameters were determined from the berry photos using the ImageJ *Measure* function. As in Ariza et al. (2010), any achene ≤ 0.4 mm in diameter was considered unfertilized (Fig. 4). Fertilized achenes were counted using Fiji's Cell Counter.

Data analysis

The differences in pollen counts between the stigma-squash and stigmagraph methods were normally distributed, so paired t-tests were used to compare the pollen load of the daffodil stigmas as determined by each stigmagraph analysis method (ICT and TWS) and the pollen load determined using the stigma-squash method. The pollen counts for each observer were first analysed separately; i.e., the ICT pollen counts done by one observer were compared to the stigma-squash counts of the same observer; we then compared the two methods using the pooled data from both observers. The pooled data were log-transformed to meet the assumption of multivariate normality and the relationship between stigmagraph pollen counts and stigma-squash pollen counts were assessed through SMA regression. The TWS was only done once by the experienced observer and was not included in the pooled analysis. The timed ICT counts and stigma-squash counts of each observer were compared using paired t-tests.

The relationship between strawberry seed set and number of pollen-bearing stigmas (as determined by Cell Counter) was modelled with simple linear regression. The number of fertilized achenes was expected to be less than or equal to the number of stigmas bearing pollen. A paired t-test was used to compare the number of pollen-bearing stigmas to the number of seeds per strawberry.

TABLE I: Comparison of the pollen counts determined by the stigmagraph processing methods and the pollen counts on the stigma squash slides for an experienced and an inexperienced observer. A positive mean difference indicates that the first method listed estimated a higher number of grains than the second method.

Observer	Compared methods	Mean difference	t	df	P
Experienced	ICT vs. Stigma squash	0.7%	0.61	21	0.55
Inexperienced	ICT vs. Stigma squash	4.0%	1.96	21	0.06
Experienced	TWS vs. Stigma squash	2.1%	1.07	21	0.30
Both (pooled)	ICT vs. Stigma squash	2.3%	1.93	43	0.06

RESULTS

The average time to process a photo of a daffodil stigma with ImageJ Colour Thresholding (ICT) and determine the pollen load was 11.7 ± 4.4 minutes (mean $\pm$ s.d.) for an experienced stigmagrapher. The average time for the same individual to count the daffodil pollen grains on a squashed stigma was not significantly different, at 11.9 ± 3.6 minutes ($t = 0.17$, d.f. = 11, $P = 0.89$). The average time for an individual new to the stigmagraph technique to photograph a stigma and process the photo was 20.2 ± 7.1 minutes, vs. only 13.42 ± 4.79 minutes for a stigma-squash pollen count ($t = 4.45$, d.f. = 11, $P < 0.001$). Thus, the inexperienced observer took significantly longer than the experienced observer to process a stigmagraph with ICT ($t = 2.96$, d.f. = 11, $P = 0.01$), whereas there was no significant difference in stigma-squash counting time between observers ($t = 0.73$, d.f. = 11, $P = 0.24$). The average time to count pollen-bearing stigmas on a strawberry flower with *Cell counter* stigmagraph was 5.8 ± 2.1 minutes. The *Cell counter* procedure was only conducted by one observer.

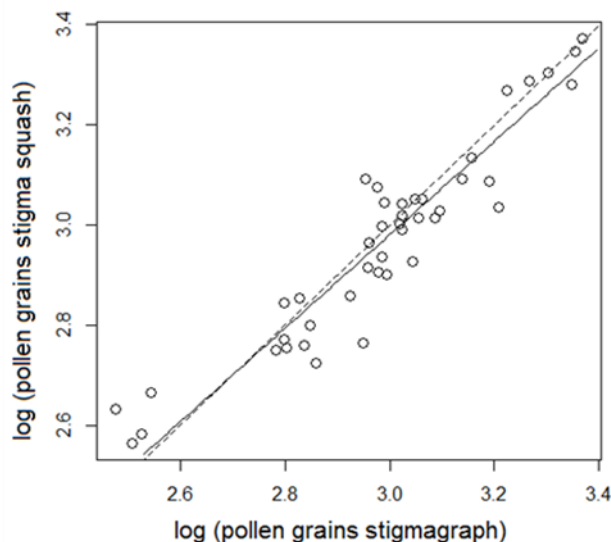


FIGURE 5. The relationship between the number of pollen grains determined by the ICT method in stigmagraphs and the number of pollen grains determined by stigma squashes for two species of daffodil (*N. pseudonarcissus* and *N. nanus*). The SMA regression line (solid) has slope = 0.93 ± 0.09 , i.e. not significantly different from 1, and intercept = 0.18 ± 0.3 , i.e. not significantly different from 0. The dotted line is the 1:1 line. $N = 44$ observations of 22 stigmas; $R^2 = 0.88$.

The stigmagraph and stigma-squash methods yielded similar stigmatic pollen counts (Tab. I, Fig. 5). The ICT method tended to estimate a higher number of pollen grains per stigma than the stigma-squash method for both observers, but this difference was non-significant for the experienced observer and only marginally significant for the inexperienced observer (Tab. I). The inexperienced observer's ICT pollen counts were, on average, significantly higher than those of the experienced observer (Tab. I).

The relationship between the pollen deposited and seed set for strawberry was analysed for 95 flowers; 25 berries were lost before harvest. There was a positive linear relationship between the number of stigmas pollinated on strawberry flowers and the number of seeds produced by those flowers (Fig. 6). The number of seeds per flower was not significantly different from the number of pollen-bearing stigmas, as determined by Cell Counter ($t = 1.11$, d.f. = 94, $P = 0.27$), but 44% of the flowers had fewer pollen-bearing stigmas than developed seeds.

For the stigmagraph test of the large stigmas of pumpkin flowers, the pollen grains were easily recognizable in the stigmagraph (Figure 7A) and readily counted using Fiji's Cell

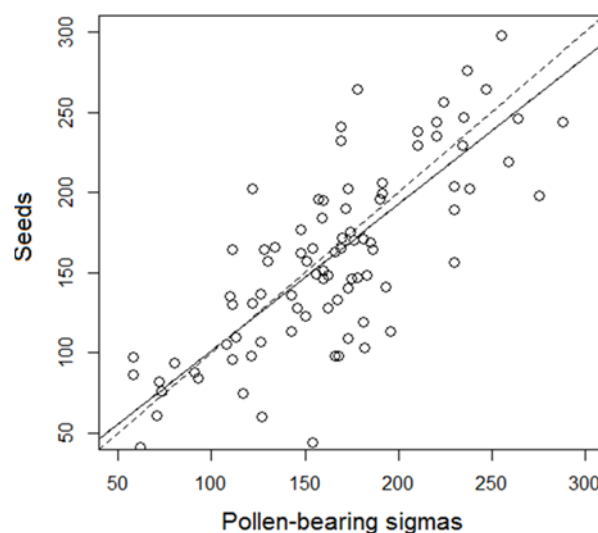


Figure 6. The relationship between number of seeds per flower and number of pollen-bearing stigmas as calculated by stigmagraphs for commercial strawberries (*Fragaria* $\times$ *ananassa*). The OLS regression line (solid) has slope = 0.92 ± 0.15 , i.e. not significantly different from 1, and intercept = 9.25 ± 26.2 , i.e. not significantly different from 0. The dotted line is the 1:1 line. $N = 95$ flowers; $R^2 = 0.61$.



FIGURE 7. Counting pollen on larger stigmas with stigmagraphs. A) A focus-stacked stigmagraph of the multi-lobed stigma of a pumpkin flower (*Cucurbita pepo* L.). B) A section of the stigmatic surface with the individual pollen grains counted using Fiji's *Cell counter*.

Counter (Figure 7B). Although the photographing time was not recorded, it takes more time to photograph the entire surface a large, multi-lobed stigma like that of pumpkin, than smaller or less-complex stigmas.

DISCUSSION

The stigmagraph method produces stigmatic pollen deposition measurements similar to those of the traditional stigma-squash method. The stigmagraph method does not necessarily reduce pollen-counting time compared to the stigma-squash method, but this new method is certainly a feasible way to determine pollen load when preparing a stigma squash is not possible—for instance, if stigmas are too large (e.g., *Cucurbitaceae* spp., *Lilium* spp.) or too numerous (e.g., *Fragaria* spp.). Pollen grains on large stigmas can be counted individually by taking a series of photographs focused throughout the depth of the stigmas. The underside or obstructed sections of a stigma can be captured by

adjusting the camera angle or removing the petals. However, manipulating floral structures may affect the behaviour of flower visitors and the problem of capturing pollen grains that are completely masked by other grains will remain regardless of camera angle. Further testing of the stigmagraph method is needed to test the accuracy of this pollen-counting technique for stigmas with various degrees of complexity. The stigmagraph method may prove capable of quantifying pollen deposition on flowers of many other plant families, such as *Cactaceae*, *Iridaceae*, or in orchids lacking pollinia (e.g. *Cypripedium* spp.).

The time required to process a stigmagraph and the accuracy of the pollen count is highly dependent upon user experience. Once a user gains experience with the ImageJ software environment, the time it takes to calculate pollen load can be less than that of a stigma squash, especially if the stigma contains a large number of grains. The accuracy of the stigmagraph method depends on the observer's ability to capture focused, high-resolution photographs of the sample flowers, which requires some practice. If the image contains aberrations due to the camera flash or light reflections, it is harder to correctly distinguish pollen from background surfaces during image processing. The 'Remove outliers' function is one option within ImageJ that can be used to reduce reflection effects (see Ferreira & Rasband 2012), but it is preferable to avoid image irregularities at the photo capture stage. Once the photography portion is mastered, this method is quick and suitable for field situations.

The accuracy of the stigmagraph method also depends on the user's ability to correctly define what constitutes pollen on the photos of the stigmas. This subjectivity introduces the potential for measurement bias, and for within- and between-observer variation in pollen counts. This subjectivity can be reduced by using the TWS tool, but if the illumination varies across stigmagraphs, pollen can be erroneously selected by the TWS as well. However, there is no reason to believe that error associated with the stigmagraph method would be more than that of the stigma-squash method, as the stigma squash involves manually counting pollen grains under a microscope, subsampling, or using similar image analysis software to determine pollen loads.

A problem with both the stigmagraph and stigma-squash methods is that pollen grains often clump together and can be deposited in several layers. Only the topmost layer of pollen can be seen in a stigmagraph. Squashing a stigma may redistribute the grains in a more uniform plane, but there can often still be a great deal of pollen clumping on a stigma-squash slide. Surprisingly, the pollen load determined on the daffodil stigmagraphs here was usually higher than that of the pollen counts on the stigma squashes. This discrepancy was most likely due to some non-pollen image aberrations being classified as pollen by the ICT. It was noticed that the reflection of light in the secreting papillae of some of the daffodil flowers was often classified as pollen by the ICT and the TWS methods. This was corrected for in most of the ICT and TWS iterations done by the experienced observer, but not by the inexperienced observer for the ICT counts.

A 1:1 relationship was expected between the number of pollen-bearing stigmas and the seed set of strawberry, but some variation is to be expected due to post-pollination processes such as ovule abortion. Although seed set is largely dependent on successful pollen deposition, the viability and successful germination of the deposited pollen can be affected by climate and environmental factors (Żebrowska 1995; Ledesma & Sugiyama 2005). Florivorous insects can also damage reproductive organs post pollination (Howitt et al. 1965; Ariza et al. 2012). These factors may explain some of the variation in the number of stigmas pollinated versus seed set, as western flower thrips (*Frankliniella occidentalis*) and tarnished plant bugs (*Lygus lineolaris*) were observed on the sampled flowers. However, the intercept of the regression of seed set on pollen-bearing stigmas was positive, indicating that more seeds were produced than would be expected based on pollen deposition (at least at low levels of pollen deposition). The higher number of seeds than pollen was most likely due to autogamous pollen that was deposited after the stigmagraphs were taken, as strawberry is self-compatible and the stigmas can remain receptive for 3–7 days (McGregor, 1976). The flowers were not emasculated and final stigmagraphs were taken two days after the flower visits, so it can be assumed that the anthers of some of the sampled flowers still contained pollen.

There are several other limitations to consider when attempting to use the stigmagraph method for pollen deposition measurement, namely, the colour of the pollen grains, the size of the stigmas, and computational power. Segmenting the photos can be difficult (and sometimes impossible) when the colour of the pollen is similar to the colour of the stigmatic surface. It can take a great deal of adjusting and readjusting to reach the correct threshold values using ICT (described above). The TWS readjusts automatically but it requires time to train the classifier on complex photos. The photos needed for this method are intrinsically large and the time required to complete the TWS on an image is highly dependent on computational power. It is recommended that 16 gigabytes or more of memory be dedicated to ImageJ to hasten photo processing. Several test stigmagraphs should be taken and processed to ensure that pollen can be distinguished from the background of the stigma prior to using this method for research purposes.

The stigmagraph method is currently not suitable for flowers with tiny reproductive structures. It was not possible with the camera lens used in this study (Canon© EF 100 mm macro) to obtain images suitable for analysis of very small stigmatic surfaces. The smallest stigmas successfully tested here were those of commercial strawberry, which are approximately 0.8 mm in diameter. Further testing with lenses of higher magnification capabilities is required to determine if the stigmagraph method can be used to measure pollen deposition on minuscule stigmas. It is presently more advisable to use the traditional stigma squash technique to determine the pollen load on stigmas smaller than 0.8 mm. For stigmas of larger size and depth, it would be necessary to use a focus-stacked set of photos to capture the majority of the stigmatic surface. Focus-stacking requires more time at each flower in the field but does not add much time to the

image-processing stage. Fiji and ImageJ are well equipped to deal with photo stacks (see Ferreira & Rasband 2012). Lastly, the stigmagraph method partitions the pollen deposited by the colour of the pollen grains. If many different co-flowering plant species are present in an area, a stigmagraph cannot discern the species identity of the deposited pollen grains, or whether or not the deposited pollen is viable.

Nonetheless, the stigmagraph method is a viable means of measuring stigma pollen load and was successfully used here to obtain pollen counts similar to those of the stigma squash method. Most notably, the stigmagraph method allows the sampled flower to remain in the field without manipulation to develop into fruit and seed. This enables a direct comparison of pollinator performance to fruit and seed set, without manipulating the sampled flower. Further, measurements of the pollen deposited by multiple successive flower visitors can be obtained using this technique, allowing the determination of each species' contribution to the stigmatic pollen load. The stigmagraph method also has the potential to track the location of pollen placement on flower stigmas, and the rate of pollen removal from stigmatic surfaces, especially in flowers with larger stigmas and pollen grains. Thus, this new method of pollen deposition measurement has strong potential to advance the field of pollination ecology.

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