

# Flow System for Automated Analysis of Maize Pollen

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Pollen grains are haploid gametes of uniform shape and size, and can be obtained in large quantity. If appropriate traits are used, they can be an excellent material for investigation of rare but important biological events like intracistronic recombinations or mutations induced by very low level of mutagens. This advantage will be further improved, if the laborious counting and examination can be made automatically. For automation of pollen analysis, techniques of flow analysis and image analysis would be applicable. Flow analysis with an optical detector was tested using maize pollen.

Pollen grains were transported by gentle suction through a glass capillary which was placed under a microscope. Interruptions of the light path by pollen grains were detected by a silicon photocell after optical magnification and converted into electric pulses. The frequency distribution of pulse height was examined by a multichannel pulse height analyzer.  $10^6$  pollen grains would be counted and classified within about 30 min for a pollen suspension dilute enough for separation of each pulse. The flow system tested seems promising for detection of *Wx* mutant pollen in a *wx* pollen population after iodine staining if illumination of sample particles is improved.

## Introduction

Pollens of higher plants are haploid and can be handled in large numbers. If appropriate genetic markers can be used, they seem to be good material for investigation of rare but important biological events like intracistronic recombination (1-3) or mutation (4-6). Since pollen grains are uniform particles in size and shape, analysis by electronic instruments would seem possible. The advantages of use of pollen would be further improved if the laborious and time-consuming procedure of scoring of pollen could be replaced by automated analytical instruments. Such instrumental analysis would be useful both in counting very large numbers of pollen and in objective classification of characters of pollen grains.

Two types of measurement can be expected, i.e., flow system and image analysis. Both systems may be used as total examination or as sampling analysis. In the flow system, however, basically all the particles are counted and examined, one by one, when they pass through the detector. The simplest

instrumentation may be a combination of particle detector and counter. If the characteristics of the particle can be classified into groups by the nature of the signals which were generated at the detector when the particle passes, addition of counters and signal analyzer will complete a basic measuring system. One such system was assembled to examine pollen grains of maize (Fig. 1). The present paper reports the preliminary results of an automated flow analysis system and makes practical evaluation for further improvements.

## Materials and Methods

### System Set-up

The flow system used is shown schematically in Figure 2. Pollen samples were suspended in water and agitated by a small stirring blade. They were transported through a glass capillary (about 0.8 mm diameter) by gentle suction of air using a microtube pump. The capillary was fixed between a slide glass and cover slip, and the spaces around the capillary were filled with balsam. The capillary was positioned precisely in the light path of a microscope. Interruptions of the light path by pollen grains

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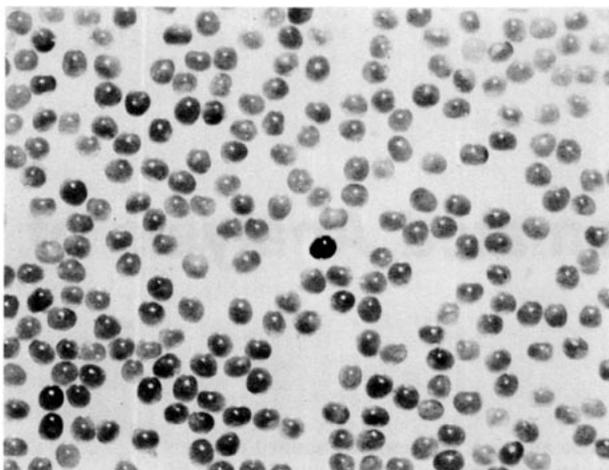


FIGURE 1. Pollen of waxy maize stained with iodine. In this material, the darkly stained pollen grain at the center is a wild type ( $Wx$ ) recombinant. Phenotypic revertant toward non-waxy may be comparable to this dark pollen grain.

were detected by a silicon photocell (Hamamatsu TV, S876-16BR) after optical magnification of about  $\times 10$ . Electric pulses from the silicon photocell were amplified and sent to a multichannel pulse height analyzer (Canberra, Series 30). The multichannel analyzer (MCA) used was capable of storage of data up to  $10^6$ -1 counts in each of its 1024 channels. It also had regions of interest (ROI) function with integrated data readout. Each input pulse was assorted to corresponding channel according to its pulse height. The number of assorted pulses was recorded in counters of each channel. Counts of neighboring channels could be pooled by ROI function by designating the region of interest. The frequency distribution of pulse height could be displayed on a CRT screen of the MCA while collecting and analyzing the data. This display could be transferred onto paper by use of an X-Y plotter. Numerical data could be printed out by a line printer. The X-Y plotter and line printer complete the instrumental set up. Extra care was taken to stabilize the light source to illuminate the capillary. Mechanical fixation, soldered leader wires, and an electronic voltage regulator were used.

## Sample Preparation

The present experiments were intended to test the flow type automatic analysis for detection of phenotypic reverse mutation of waxy ( $wx$ ) pollen to non-waxy ( $Wx$ ) pollen. For this purpose, mature maize pollen was used to test the flow system and to obtain optimal flow rate and other operational data.

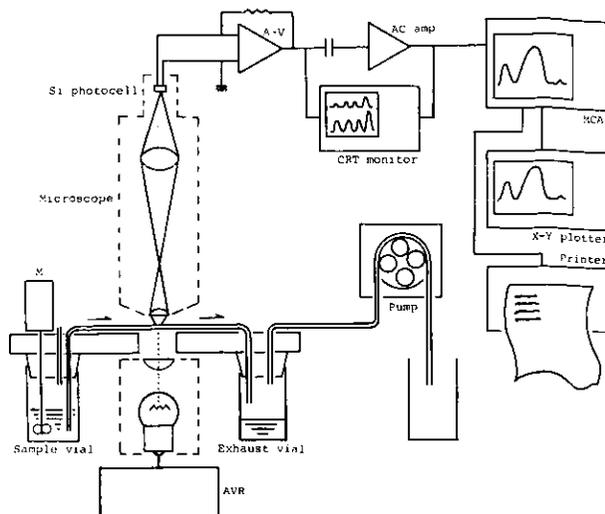


FIGURE 2. Schematic diagram of instrumentation of the flow system. Pollen suspension is transferred through glass capillary by a microtube pump. Interruptions of light path by pollen grains are detected by a silicon photocell. Electric pulses are amplified to a multichannel analyzer (MCA). MCA classifies each pulse according to its pulse height. Collected data can be transferred to an X-Y plotter or printer for recording.

Mature maize pollen had been harvested in the field and stored in 70% ethanol. Some younger pollen usually used in fine structure analyses of  $wx$  locus, was also tested after iodine staining (Fig. 1). In both cases, pollen grains were washed clean with 70% ethanol a few times to eliminate small debris in the sample suspension.

## Pulse Shape Analysis

Shapes of the electric pulses generated by interruptions of light by pollen grains were brought to a standstill for observation on a CRT screen of a dual channel synchroscope (Iwatsu Electric, Synchroscope SS-5050) using digitized memory (Kawasaki Electronica, Transient Memory TM-1410). The Transient Memory had outputs for an X-Y plotter to make a paper copy of pulse shape. To the second channel of the Synchroscope, either calibration voltage for pulse height or timing pulses for pulse width could be applied for measurements.

## Measuring Procedure

After a 1 hr warmup to stabilize the instruments, the sample vial and exhaust vial were set in position. The agitator of the sample vial and microtube pump for transportation were turned on. When the grains start moving through the capillary, electric pulses were confirmed on a monitor

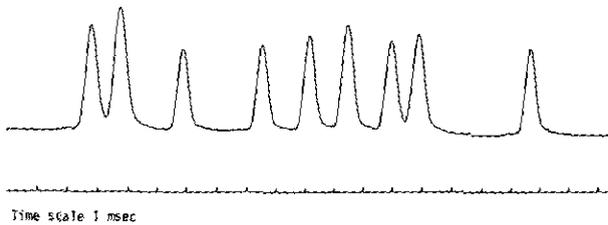


FIGURE 3. Pulse shapes of pollen counter. Nine pulses were recorded for 20 msec.

Pulse shape of pollen counter (piled up by too many pollen).

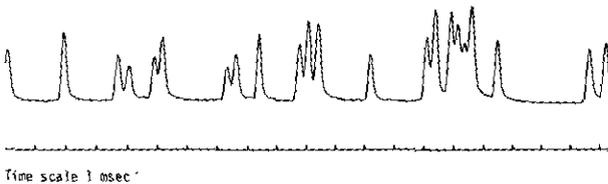


FIGURE 4. Pulse shape of pollen counter when concentration of pollen suspension is high. 21 pulses were recorded for 20 msec.

Multi Channel Analyzer out put.

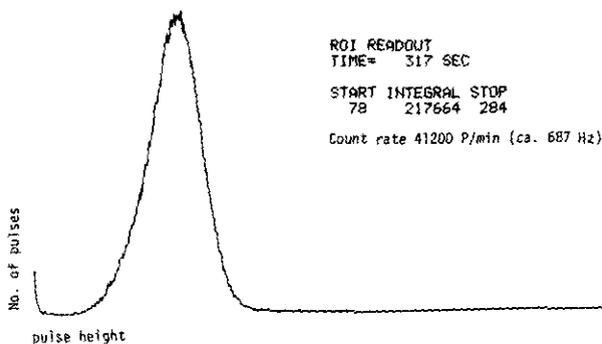


FIGURE 5. Frequency distribution of pulse height. Region of interest was set from channel 78 through channel 284 of 1024 channels of the MCA to include the main peak.

synchroscope (Matsushita Communication Industrial, VP-5102A). MCA was activated for data collection by turning on the "collect" switch. When most of the sample suspension was transported, and before air bubbles came up, data collection by MCA was terminated by turning off the "collect" switch. ROI was set after data collection to include the major part of the peak of frequency distribution of pulse height. The X-Y plotter and digital printer recorded results of analysis.

## Results

### Flow System

Although the capillary used for maize pollen grain was quite large, particles flowed at the center of the capillary when suspension was transported at the rate of 2 ml/min or 4 m/min. This centering of pollen grains in flow was an unexpected advantage of the present system and made focusing of pollen images on the photocell easy. Clogging in the flow system could be avoided by use of a thick capillary and cleaned pollen suspension. However, the cleaning of the pollen suspension eliminated both small debris and empty shells of abortive pollen. The latter fraction would be biologically important in some experiments, but in the present experiments only fully grown pollen grains were analyzed.

### Pulse Shape

The silicon photocell used had a narrow light-sensitive area of 1.2 mm × 6 mm, and was placed to transverse the image of the capillary. This gave a sharp pulse and high resolution. Pulse shapes were uniform with little variation in pulse height when a diluted pollen suspension was used. Examples of the pulse shapes are shown in Figure 3. The count rate for this concentration was 500 pulses/sec or  $3 \times 10^4$  pulses/min. Clustering or clumping of the particles occurred when the concentration of suspension was higher. An example of such piled up pulses is shown in Figure 4.

### Frequency Distribution of Pulse Height

Electric pulses were generated by interruption of the light path by pollen grains. The height of each pulse reflects the characteristics of the pollen, the optical density, which might relate to size and color of pollen grain. The size of pollen grain was also expressed as width of each pulse. In the present experiments, the size of pollen grains did not vary very much by visual examination. The frequency distribution of pulse height was analyzed by MCA. An example of the frequency distribution of pulse height of unstained mature pollen of maize is shown in Figure 5 together with ROI readout data. In case of piled up pulses like those in Figure 4, the peak of the frequency distribution of pulse height was broad. A mixture of younger pollen was also examined after iodine staining, but, as discussed later, pulse heights did not differentiate between  $Wx$  and  $wx$  as far as transmitting light was used.

## Discussion

Automation of the pollen analysis may be worthwhile both for quick and accurate counting of the total number of pollen grains and in objective classification of the character of pollen. If well cleaned maize pollen were to be used, counting of the total number of pollen grains would be easy with the flow system reported here and a low cost electronic counter. Counting of pulses which exceeded in their height the predetermined threshold level could be made by using a discriminator or comparator circuit. Recent advancements in the electronic industry have developed a compact and relatively low cost multichannel pulse height analyzer (MCA). The MCA used here had 1024 counters, each capable of  $10^6$ -1 counts, and was able to sort pulse to the corresponding counter according to pulse height. This could be used as main analyzer of the flow system.

Theoretically, four classes of pulses, each differing in height, would be expected in reversion experiments of waxy maize pollen if optical detection of particles was used. They would be, in order of increasing height, (1) small debris and noises inherent to the elements used, (2) abortive pollen, (3) normal pollen, and (4) mutant *Wx* pollen. As described before, the height of the pulse might reflect optical thickness and color of the pollen grain. The size of pollen grains did not vary significantly, but as maize pollen grains were egg-shaped and the directions of their axes might vary randomly in flow, the optical thickness as sensed by the photodetector might deviate considerably. However, the difference in color of typical *Wx* and *wx* pollen after iodine staining was unmistakably clear. *Wx* pollen stains dark blue-black and *wx* light brown. For objective classification of pollen character, use of an appropriate color filter will help to differentiate mutant *Wx* pollen from parental *wx* pollen.

Illumination of pollen grains was important. In the case of *wx-Wx* pollen experiments, *Wx* pollen can be detected best with reflecting light after iodine staining. However, if only reflecting illumina-

tion were used with a dark background, darkly stained *Wx* pollen would produce lower electric pulses than pulses produced by light colored *wx* pollen. This would make distinguishing between darkly stained mutant *Wx* pollen and empty shells of abortive pollen difficult. Improvement is possible by adopting both types of illuminations. Transmitting illumination would produce an electric pulse which corresponds to each pollen grain, and reflecting illumination would permit subtraction of the height according to lightness of color. This would leave pulses from dark *Wx* pollen less affected and maintaining their height highest among the four classes of pulses described before. An additional red color filter would help in differentiating mutant *Wx* pollen pulses from background *wx* pollen pulses.

It should be mentioned that in the present set-up, sample and exhaust vials were exchangeable and pollen was intact after examination. Pollen could be counted or examined repeatedly for the total number and presence of mutant pollen grains or could be spread on a large slide glass for visual examination.

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