Chapter 25

Impedance Flow Cytometry as a Tool to Analyze Microspore and Pollen Quality

Iris Heidmann and Marco Di Berardino

Abstract

Analyzing pollen quality in an efficient and reliable manner is of great importance to the industries involved in seed and fruit production, plant breeding, and plant research. Pollen quality parameters, viability and germination capacity, are analyzed by various staining methods or by in vitro germination assays, respectively. These methods are time-consuming, species-dependent, and require a lab environment. Furthermore, the obtained viability data are often poorly related to in vivo pollen germination and seed set. Here, we describe a quick, label-free method to analyze pollen using microfluidic chips inserted into an impedance flow cytometer (IFC). Using this approach, pollen quality parameters are determined by a single measurement in a species-independent manner. The advantage of this protocol is that pollen viability and germination can be analyzed quickly by a reliable and standardized method.

Key words Impedance flow cytometry, IFC, Microfluidic chip, Pollen viability, Developmental stages, Pollen germination, Pollen size, Label-free

1 Introduction

Sexual plant reproduction either for maintaining genetic diversity, seed or fruit production relies on viable and functional pollen grains [1]. Off-season crossing or germplasm preservation programs benefit from conserved and stored mature pollen that retain their germination capacity [2]. Immature pollen of specific stages (microspores) are used to produce homozygous parental lines for breeding purposes [3]. However, genetic and environmental factors affect pollen development and viability [4, 5] and consequently reproduction [6–9]. Therefore, an estimation of pollen quality parameters like developmental stage, viability, and germination capacity is crucial for successful pollen-based processes.

Currently, various methods like staining with fluorescent/non-fluorescent dyes (stage and viability) [10, 11], size measurements (ploidy) [12], or in vitro pollen germination are used to determine pollen quality. However, these methods have disadvantages with

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respect to reliability, analysis time, labor intensity, genotype and species dependency. Moreover, the estimated pollen viability does often not correspond to pollen germination even when the correct protocol has been applied [13, 14]. Recently, a general, efficient, and reliable method that determines pollen viability and predicts germination using microfluidic chips in combination with an impedance flow cytometer has been described [15].

Impedance flow cytometry (IFC) is based on the electric properties of cells that react to an applied alternating current (Fig. 1). By varying the frequency (MHz) of the alternating current information about cell size, membrane integrity and cytoplasmic conductivity are simultaneously obtained. IFC has been used in microbiology and human cell cultures to characterize cell types [16] and culture conditions [17], but has not been widely used for plant cells [15].

Here we describe a detailed protocol to follow pollen development, analyze pollen viability, and predict pollen germination using IFC [15]. In the described method microspores, immature or mature pollen grains are isolated or harvested, resuspended in specific measurement buffers, and filtrated to remove particles that are larger than the actual pollen grains to prevent clogging of

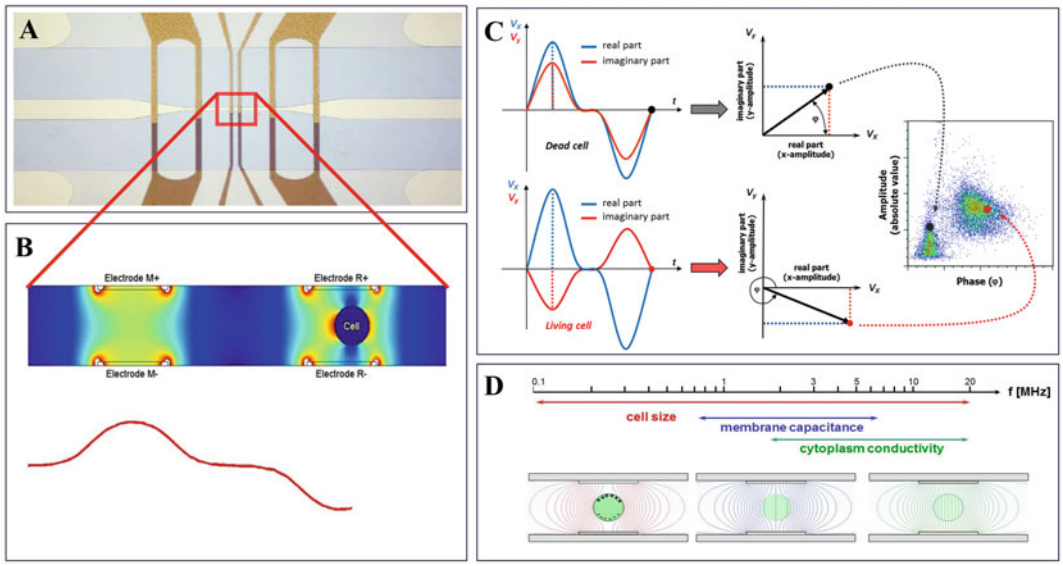

Fig. 1 Working principle of IFC. The microfluidic chip (**A**) consists of a sensing channel whose width can range from 50 to 250 μm, as well as measurement "M" and reference electrodes "R," to which an alternating current is applied (**B**). As a cell or particle crosses the electric field, it changes the impedance value of the liquid in a characteristic way. The impedance signal is split in its real and imaginary components (v_x and v_y), which are different for dead and living cells (**C**). A smaller and more compact (dead) cell gives a smaller signal in both amplitude and phase angle as a bigger and physiologically active one (**C**). According to the dielectric properties of cells these differences can be observed at higher frequencies, where membrane capacitance (integrity) and cytoplasmic conductivity are interrogated (**D**)

the microfluidic chip. The sample is then loaded into an impedance flow cytometer with default electronic settings of trigger level, amplification, and demodulation. As the measurement proceeds, the data of the analyzed cells are displayed in amplitude/phase angle dot plots and related histograms by which the cell/pollen populations are characterized. Non-developing microspores and dead pollen grains are usually reduced in amplitude value and phase angle while viable cells show a slightly higher amplitude value and a distinct higher phase angle [15]. Pollen with germination capacity are a part of the viable populations that show a higher phase angle than the non-viable ones. By adapting the electronic setting, the pollen population with germination capacity can be discriminated from the viable and non-viable ones [15]. The different populations are identified by inactivation controls; the populations that disappear after the treatment must have been the viable and/or germinating ones, as the cells are not harmed or destroyed during the measurement [15].

This simple method opens opportunities to screen for any genetic or environmental effect affecting pollen development, viability, and germination in a reliable and standardized manner. As the cells are not harmed during the passage through the IFC, the analyzed microspores or pollen can be recollected and used for further experiments or applications.

2 Materials

Most of the required equipment can be found in standard cell or molecular biology labs.

2.1 General Equipment

1. Water purification system to produce deionized water (15–18 MΩ, Millipore) (*see* **Note 1**).
2. Microwave, heater, water kettle, or water bath.
3. Sterile disposable pipettes of various volumes, micropipette and tips for small-volume dispensing, microcentrifuge tubes; 250 µl PCR-strips, fine forceps (Dumont no. 5 or 7).
4. PCR machine, 96-well with gradient option between 20 and 60 °C.
5. Vortex.
6. Table top centrifuge.
7. Cell strainers with appropriate mesh size, e.g., 30, 50, 100, and 150 µm (*see* **Note 2**).
8. Microscope with size measurement tool (*see* **Note 2**).
9. Glass slides and coverslips.
10. 2 ml rubber bulbs (grey) as used, e.g. for Pasteur pipettes.

Table 1
Standard pollen germination media

Component	Brewbaker and Kwack [23] (Solanaceae)	Vizintin and Bohanec [24] (Cucurbitaceae)
Sucrose	100 g/l	
Maltose		150 g/l
KNO_3	100 mg/l	
$Ca(NO_3)_2 \cdot 4H_2O$	300 mg/l	300 mg/l
$MgSO_4 \cdot 7H_2O$	100 mg/l	
H_3BO_3	100 mg/l	250 mg/l
Adjust to indicated pH (with 1 N KOH or HCl)	pH 5.2	pH 7.0

2.2 Special Equipment, Solutions, and Plant Material

1. Impedance Flow Cytometer Ampha Z30 (or derivatives) plus accessories including AmphaSoft software (Amphasys).
2. Microfluidic chips at 50, 120, or 250 μm channel size (Amphasys).
3. Waterpik FLW220 with tips or any other cordless electrical tooth flosser or brush that allows changing tips.
4. Micro pestles with tips fitting into 1.5 and 2 ml microcentrifuge tubes (preferentially the safe-lock type).
5. Analysis buffer AF 1–6 (Amphasys) or appropriate (*see* **Note 3**).
6. 2% (w/w) Tween 20 diluted in AF buffer.
7. 20% (w/v) potassium chloride solution (KCl).
8. Polystyrene beads of various sizes 10–100 μm (Sigma) (*see* **Note 4**).
9. Pollen germination media (examples of standard compositions are given in Table 1).
10. Gene frames (125 μl).
11. Pollen from plants either grown in the field, greenhouse, or controlled growth chambers as appropriate for the species and cultivar (*see* **Note 5**).

3 Methods

The method of pollen collection and subsequent sample preparation can vary slightly depending on the species and type of analysis. The typical workflow comprises pollen collection, extraction, filtration, and measurement (*see* Fig. 2). The identification of dead, non-germinating, and viable populations occurs by the disappearance of certain populations, the viable or germinating one, after inactivation (inactivated controls).

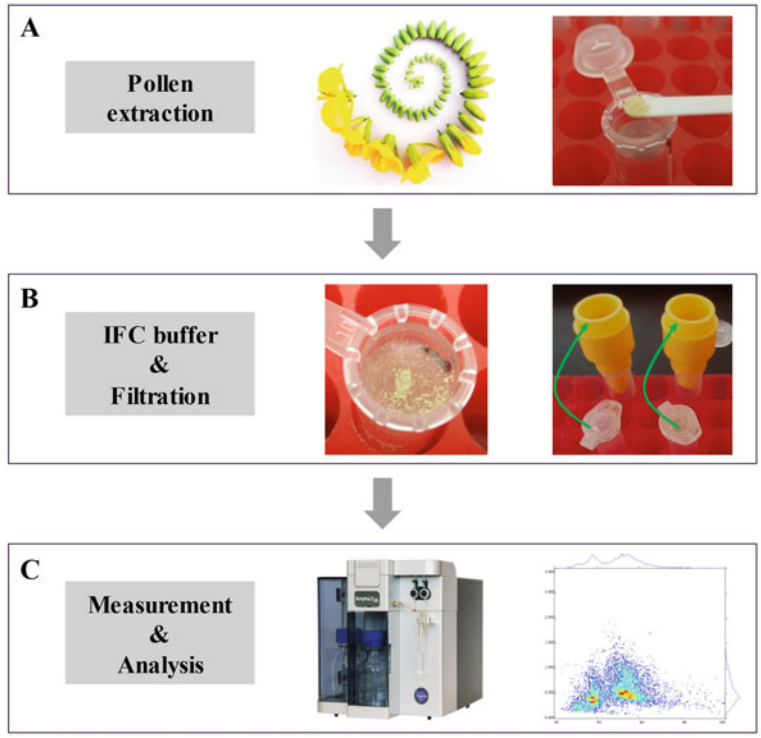

Fig. 2 Workflow. Microspores or pollen are isolated or harvested from closed buds or open flowers, respectively (**A**). Microspores or pollen resuspended in AF buffer are passed through cell strainers of appropriate size (**B**), loaded onto Ampha Z30 and analyzed (**C**)

3.1 Pollen Collection and Sample Preparation

3.1.1 Estimation of Viability and Developmental Stage

1. Collect a range of single, fresh flower buds with increasing sizes and sort them from small to big accordingly (*see* **Note 6**, Fig. 2A).
2. Place whole buds or extracted anthers into 1.5 or 2 ml microcentrifuge tubes containing 500 µl AF buffer and squeeze buds/anthers gently with a micro pestle (*see* **Note 7**).
3. Remove micro pestle, add 1 ml AF buffer, close the tube and mix the content by flicking the tube briefly or using a vortex.
4. Prepare fresh microcentrifuge tubes, insert cell strainer and pour the suspension through (Fig. 2B, *see* **Note 8**).
5. Remove cell strainer, close tube, and continue with the next sample or prepare appropriate control as described in Subheading 3.3.
6. Prepare for measurement as described in Subheading 3.4. (Figs. 2C and 3).

3.1.2 Mature Pollen Viability

1. Collect mature, dry pollen (e.g., tomato, sweet pepper, potato, wind pollinators) from open flowers by inserting the tip of the

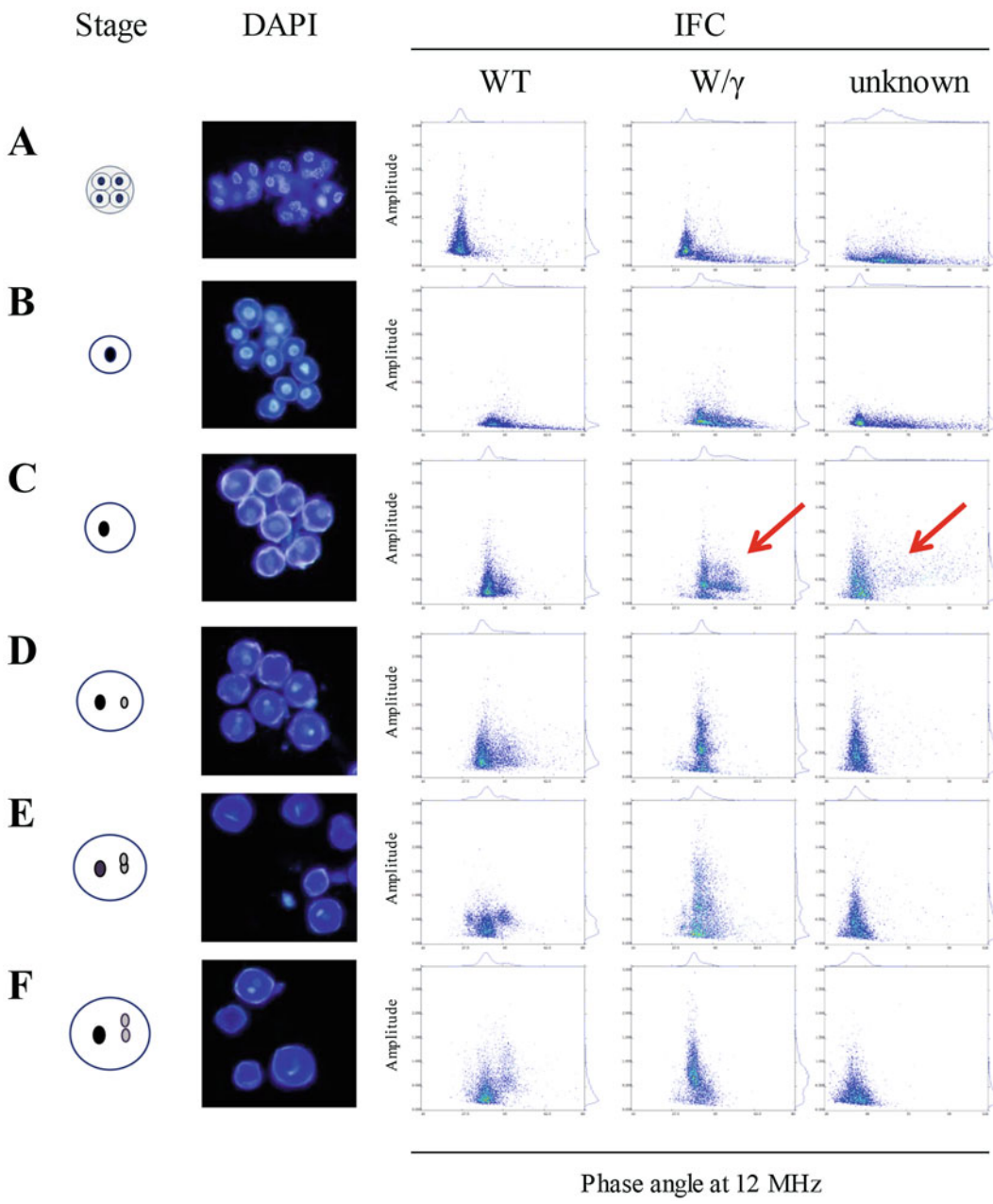

Fig. 3 Detection of developmental aberrations in potato pollen. Pollen development follows well defined steps. After release from the tetrad (**A**) the young microspore bears a central nucleus (**B**), at a later stage this nucleus migrates to the cell wall (**C**), divides to form an immature pollen grain with a generative (*gray*) and vegetative nucleus (*black*) (**D**). As the immature pollen grain matures the generative nucleus divides again (**E**) forming two sperm cells that remain attached to each other (**F**). Isolation of pollen from putative sterile plants like the potato genotype "W/y" or an undescribed sterility like in the genotype "unknown" shows that the stage of pollen abortion can be detected without DAPI staining

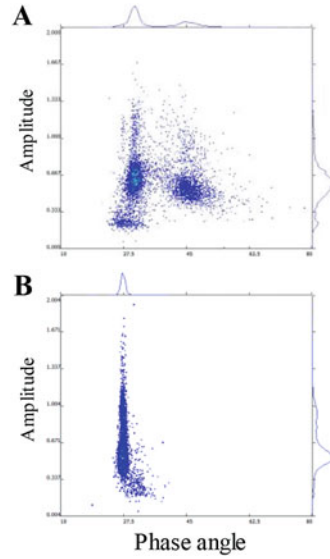

Fig. 4 Identification of dead and viable pepper pollen populations. The pollen populations are identified by heat-inactivation. Pollen populations usually display two clouds, one with a lower and one with a higher phase angle (**A**). Upon heat-inactivation the population with the higher phase angle disappears (**B**), identifying this one as the viable one that has been inactivated. The analysis was performed at 12 MHz

anthers into an 1.5 ml microcentrifuge tube and shake the flower using a waterpik, continue with **step 3** (*see* **Note 9**).

2. Collect mature, insect-transferred pollen (e.g., melon, cucumber, Brassica-species) by removing anthers from the open flowers and transfer them into 1.5 ml microcentrifuge tubes (*see* **Note 10**).
3. Add 1.5 ml of AF buffer, mix well and continue as described before (*see* Subheading 3.1.1) from **step 4** onwards (*see* **Note 11**).
4. Prepare killed pollen sample as described (*see* Subheading 3.3.1 or 3.3.2) to create a negative (dead) control sample as shown in Fig. 4.

3.1.3 Pollen Viability Combined with Germination (Tomato)

1. Collect mature pollen as described in Subheading 3.1.2.
2. Incubate pollen (fresh or dried) for 1 h in 2 ml AF6 buffer, mix from time to time.
3. Centrifuge for 3 min at 1500 × *g* in a microcentrifuge, remove liquid and resuspend in 250 µl AF6 buffer.
4. Prepare two equal aliquots of 100 µl.
5. Prepare one aliquot as germination-inactivated control as described (*see* Subheading 3.3.3).

6. Transfer the 100 µl aliquots each to 1.5 ml AF6 buffer.
7. Filtrate and measure all samples (*see* Subheading 3.4) to define the dead, viable, and germinating pollen population (Fig. 5). Verify by germination assay (*see* **Note 12**) as described below.
8. Centrifuge the remaining sample (about 250 µl) for 3 min at 1500 × *g* and remove the AF6 buffer.
9. Resuspend the pellet in 250 µl germination medium suitable for the species (*see* Table 1), spread into gene frames mounted onto glass slides and incubated for 90 min at RT in boxes with a relative humidity (RH) of about 80%.
10. Count spores as germinated when the pollen tube exceeds the diameter of the grains and calculate the percentage of germinated pollen.

3.2 Size Determination

1. Mix the original vials containing the polystyrene beads well.
2. Prepare a bead control solution for either 10, 20, or 30 µm beads by diluting the respective polystyrene suspension in AF buffer to a final concentration of approximately 5×10^4 beads/ml, and 60 or 100 µm beads to a final concentration of 1×10^4 beads/ml.
3. Add 30 µl of the diluted beads either individually or as mix (*see* **Note 4**) to each filtrated microspore/pollen sample (1.5 ml) prepared as described above (*see* Subheadings 3.1.1 and 3.1.2) and mix well (*see* **Note 13**).
4. Continue with Subheading 3.4 (measurement).

3.3 Control Sample Preparation

Dead or inactivated germination controls are essential for the identification of the analyzed populations. While temperature treatments around 40 °C inhibit pollen germination, temperatures above 54 °C completely kills pollen, at least in the tested species [15]. Based on the laboratory equipment, there are different options to create those:

3.3.1 Estimating the Killing Curve of Viable Pollen by Using a PCR Gradient

1. Prepare 1.5 ml of at least double concentrated, filtrated microspore/pollen suspension as described in Subheadings 3.1.1 and 3.1.2.
2. Fill 250 µl PCR strip vessels (12 per strip) with 100 µl of microspore/pollen suspension and close vessels.
3. Set the PCR program to a temperature gradient from 40 to 60 °C with a 15 min incubation step. Write down the different temperatures per well (*see* **Note 14**).
4. Fill 1.5 ml microcentrifuge tubes (one per treatment, tube marked) with 1 ml AF buffer.
5. Transfer the pollen suspension into the microcentrifuge tubes, close the lid and proceed to Subheading 3.4.

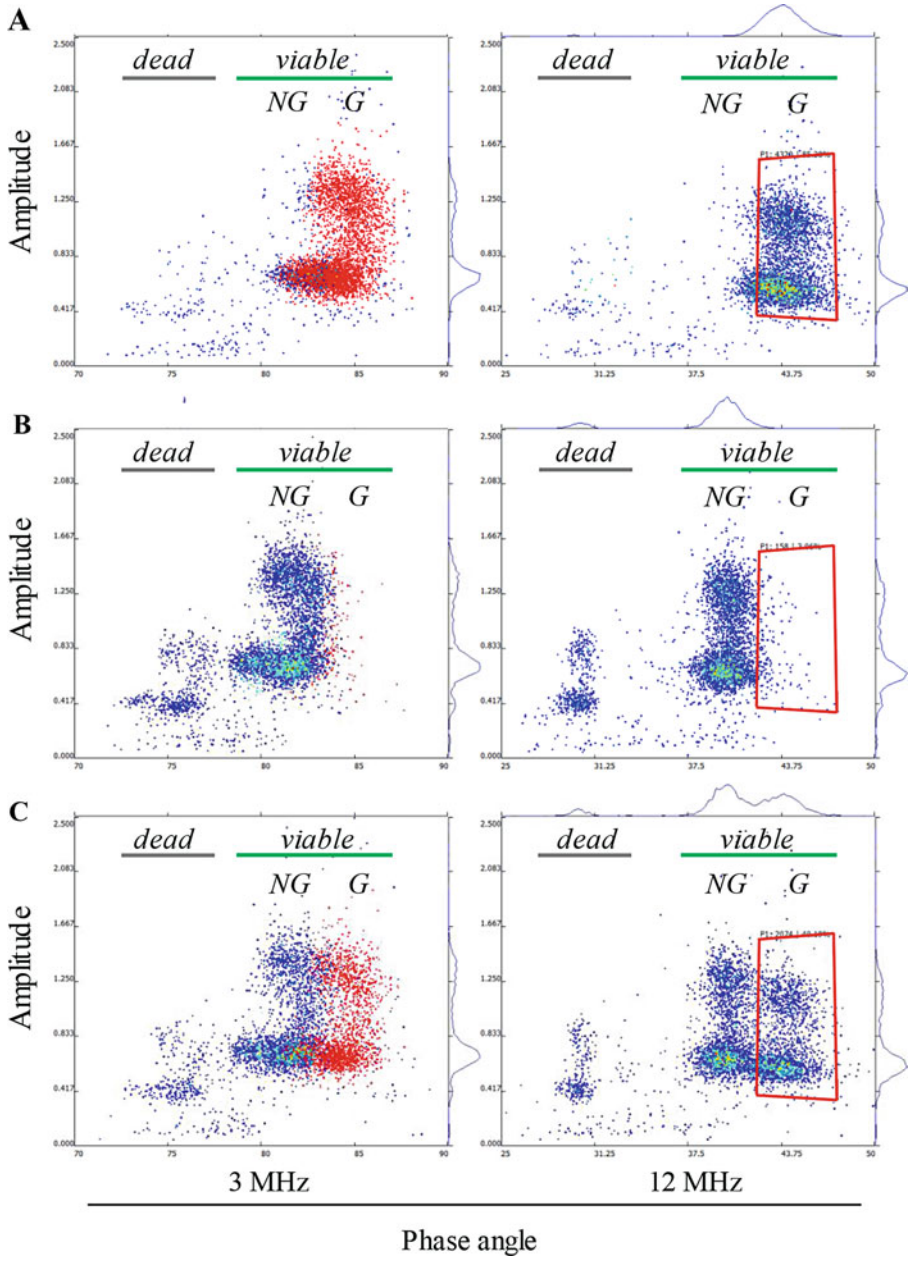

Fig. 5 Prediction of pollen germination. Pollen germination of tomato can be predicted at 3 and 12 MHz. Pollen populations usually carry dead (*grey bar*) and viable pollen grains (*green bar*) with a low and high phase angle, respectively (**A**). The viable population consists of pollen without (NG) and with germination capacity (G, marked in *red* at 3 MHz, and with a *red polygon* around the corresponding population at 12 MHz). A 15 min treatment at 40 °C inactivates in vitro tomato pollen germination but is not killing the pollen, the viable population (*blue*) is still present but the one with germination capacity is lacking (*red*) (**B**). In an equal mix of the two treatments (**C**) the different phase angle of the two populations becomes obvious

3.3.2 Killing Pollen by Hot Water Treatment

1. Place a mug (250 ml) with water into a microwave and heat up until it is cooking.
2. Remove mug and place a closed 1.5 or 2 ml safe-lock-tube containing microspore/pollen suspension into the hot water.
3. Incubate for 15 min, cool down to room temperature afterward, and proceed to Subheading 3.4 (*see* **Note 15**).

3.3.3 Inactivation of Pollen Germination

1. Resuspend a substantial amount of pollen (at least 6×5000 pollen grains) in AF buffer and incubate for 1 h at RT and filtrate.
2. Prepare samples and Eppendorf tubes as for the killing curve but set the PCR program to a temperature gradient from 20 to 40 °C with a 15 min incubation step (*see* **Note 16**).

3.4 Measurement

1. Start-up Ampha Z30 and software as described by the manufacturer.
2. Insert measurement chip and rinse it.
3. Choose measurement buffer, flush cycles between samples, and stop conditions (optional). Stop conditions allow terminating the measurement after a certain number of counted cells or a defined sample volume.
4. Before measurement adjust the stop condition to a higher cell count to visualize the beads along developing pollen grains and estimate the sizes (Fig. 6A), or to determine a dead population (Fig. 6B).
5. Choose frequencies (MHz), 0.5 and 12 MHz for viability, 12 MHz for germination capacity (*see* **Note 17**).
6. Prepare work space as described by the manufacturer.
7. Safe work space.
8. Shake individual samples before each measurement to create an equal distribution of pollen grains throughout suspension.
9. Insert the sample tubing tube into the microcentrifuge tube and start the measurements.
10. The machine will stop automatically if stop conditions are provided, without stop conditions the measurement has to be stopped manually (*see* **Note 18**).
11. After the flushing cycle proceed with the next sample.

3.5 Data Analysis

1. Adjust first the phase range and amplitude value for all samples by using the "zoom"- and "apply all" function (see manual of the supplier).
2. Compare the viable with the dead/inactivated control sample and place the gating accordingly (see manual of the supplier). Apply to all sets the same parameters for all measurements and

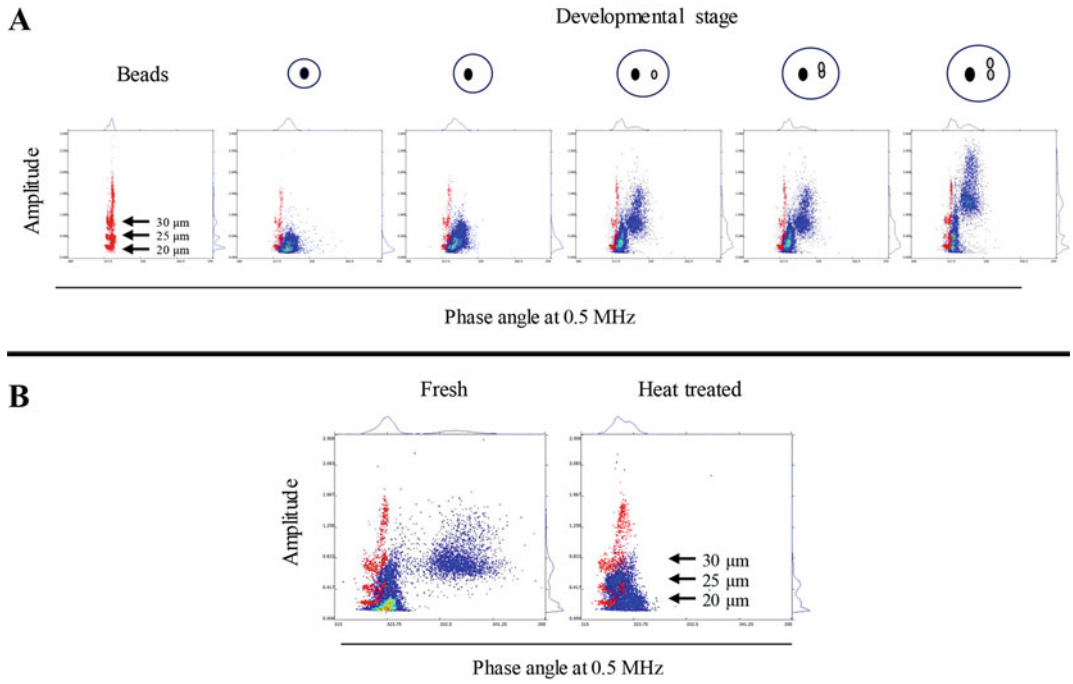

Fig. 6 Size determination by beads. In the absence of a microscope with size measurement tools a mix of polystyrene beads of different sizes (marked in *red* with size indication) can serve as marker-set to estimate the size of developing pollen (**A**) or the difference between viable and dead pollen populations (**B**) using the amplitude value at 0.5 MHz

calculates automatically the percentages of cells within the different gates (*see* **Note 19**).

3. Export collected data to excel compatible file by choosing the "cvs"- option in the report function for further statistical analysis, etc. (*see* **Note 20**).

4 Notes

1. Deionized water of 15 MΩ is the minimal required water quality to run the Ampha Z30 machine. If no system to produce water of the required quality is available, aqua dest. purchased at pharmacies or other shops can be used.
2. The use of cell strainers is essential as pollen samples usually contain debris, like trichomes, hairs, pollen aggregates, or small insects, which has to be removed prior to measurement. Omission of this step results in chip clogging and failure of the measurement. The cell strainer type is chosen in relation to the used chip and pollen size. The use of a mesh size smaller than the standard pollen size will not result in a representative sample. It is recommended to estimate the pollen size first,

either by measuring under a microscope (e.g., cell sense software Olympus), or consulting suitable websites (www.amphasys.com; http://pollen.tstebler.ch/). Alternatively, use the cell strainers to estimate pollen size by passing a suspension through a tower of different mesh sizes decreasing from 150 to 20 or 10 μm, observe the formation of fractions, and check them under the microscope. Non-developed and dead pollen is usually of a smaller size than viable pollen.

Cell strainers can be re-used after thorough cleaning and complete drying. Salt or water residues within the mesh can affect the conductivity of the buffer and interfere with the measurement.

3. The AF buffers are designed per pollen type and species. They are used as extraction and measurement buffer. As a general rule, dry pollen is best to be measured in AF4 or AF6, while AF3 or AF5 are optimal for insect-transferred, sticky pollen. A recommendation for a number of species is given at the supplier's website (www.amphasys.com).

   For the analysis of pollen development (microspore stages), the standard isolation buffer can be used. When adjusting a non-AF medium or buffer the liquid impedance has to be measured and if necessary adjusted to the optimal value designated per chip by the supplier (www.amphasys.com). It is recommended to adjust the liquid impedance with potassium chloride, e.g., the standard isolation medium for *Brassica* microspores (1/2 B5 medium [18], 13% sucrose) for an optimal separation of the dead and viable population.

   AF buffers contain sugars and should be handled under sterile conditions, or otherwise be used in small aliquots that can be used in one day.

4. The use of polystyrene beads is an option. They are delivered at specific sizes and can be mixed in certain ratios and combination, depending on the experimental design and the expected size of the pollen grain. Of course, beads can also be used as size indicator using a microscope. The beads will be visible as round spheres and with a different reflection under a light microscope. As an example, tomato pollen have a size between 20 and 25 μm, thus polystyrene beads between 15 and 30 μm are of appropriate sizes. Beads of 60 or 100 μm are suitable for bigger pollen like cucumber or corn (50–90 μm). When beads have been added to the samples, two extra rinse cycles should be applied after the measurements to thoroughly clean the fluidic tubing.

5. It should be considered that pesticide treatments, pests, or extreme temperatures might affect pollen quality [19]. For certain screenings, like for cytoplasmic male sterility (CMS), repetitive measurements are recommended, as CMS can be

influenced by environmental conditions [20] or even mimicked by those [19].

6. Remove any open flowers sheading pollen to avoid contaminations of the other samples with mature pollen. In many species, pollen development is accompanied with an increasing bud length. For a reliable analysis, it is recommended to measure the buds length by a standard ruler. For a precise stage determination, uni-nuclear to tri-cellular, a standard DAPI staining is recommended. The described technique allows a quick screening, e.g., of disrupted pollen development or a rough state determination.
7. Some flower buds are relatively sturdy or carry a big ovary, like sweet pepper or onion, which hampers the extraction of the microspores. Other flowers release quickly oxidizing compounds when wounded, like Florence fennel, which affect pollen viability. In these cases and in cases where buds carry a lot of hairs/long trichomes an anther extraction using fine forceps is recommended. Buds from *Brassica* species are soft with a small ovary and can easily be squeezed as whole.
8. Pouring the microspore suspension is the fastest option. The suspension can also be pipetted if it does not contain too much debris which hampers pipetting. Cell strainers can have some air within the mesh, a quick lift and drop of the strainer onto the microcentrifuge tube releases the captured air and allows the filtration. Filtration can further be enhanced by placing a 2 ml (grey) rubber bulb tightly onto the strainer (fits exactly onto the Celltrix cell strainers) and pressing it gently.
9. It is recommended to change the tip of the waterpik frequently or per plant to avoid pollen contamination and/or disease spreading.
10. While melon and cucumber anthers can be best collected by using a Dumont No. 7 forceps, Brassica-anthers with their relatively long filaments can be best pinched from the open flowers by placing the anthers between the opening and the lid of the microcentrifuge tube and just closing the lid.
11. Stored pollen is treated as described for mature and dry pollen. It depends on the species [21] if a hydration phase is necessary or not.
12. The in vitro germination assay in this case serves as a control when this measurement is executed for the first time. It has to be taken into consideration that not all described pollen germination protocols/media are optimal for each genotype/species, which might influence the strength of the correlation between IFC and in vitro germination data. For convenience, the compositions of two standard germination media are given in Table 1. However, additional work prior starting these

experiments is likely needed to find the optimal germination medium if the standard media are insufficient to initiate a pollen germination of at least 50%.

13. It is recommended to prepare two controls, a pollen-free bead and a bead-free pollen sample, because the beads will show no change in phase and can disguise the dead pollen population. The amount of beads added to a pollen suspension should be about equal to the amount of pollen to avoid an overrepresentation of beads.
14. Automatic gradient calculations of PCR machines do not increase the temperature/well by a fixed value. If the temperatures of two adjacent wells are too close together, like 40 and 40.5 °C, it makes sense to skip one or two wells to have a bigger temperature difference between the treatments, e.g., 40 and 42 °C.
15. This is the fastest and most easily applicable killing method. It is usually applied after the standard measurements and especially in cases when not much experience with a certain pollen type has been gained previously. The best method to fix the microcentrifuge tube in the mug is to put a piece of tape onto the lid of the closed tube and attach the ends across the open mug which holds the tube down into the hot water. Within 15 min the water temperature of the mug (at room temperature) drops from 90 to 60 °C which is sufficient to kill the pollen. A water bath or lab-heater (with microcentrifuge tube block) set to 60 °C can also be used for the 15 min treatment.
16. All pollen populations that we have analyzed so far and that were subjected to the treatment above 37–40 °C did not germinate. However, it cannot be excluded that pollen of certain species used to a climate around or above this temperature range are tolerant to these temperatures. For those species, the PCR gradient has to be adjusted accordingly.

    It is possible that germinating pollen of different species react differently and the identification of the germinating population has to be worked out by adjusting the default settings and the use of optimized AF measurement buffers for each species.
17. In most species, the discrimination between dead and viable is sufficient at 0.5 MHz but in some cases, as for the estimation the germination capacity, the measurement at 12 MHz provides more detailed information. For specific measurements, the default setting of the machine has to be adjusted in the expert window.
18. For most pollen measurements, 3000–5000 analyzed cells are sufficient for a reliable interpretation. When beads were added to the pollen sample, it is recommended to either switch off the stop condition “cells” or to set the stop condition to

8000–10,000 cells. Beads analyzed by the 120 μm chip at 0.5 MHz but not at 12 MHz can appear in a "J"-shape. This is due to the variation in size of the beads and their position within the channel as they pass the measurement electrodes, an effect described as inertial focusing [22].

19. Specific populations can be marked and named by using the option "polygon-gating" and "select cells" (see manual of the supplier).
20. Alternatively, an html-file which contains all data and the images can be created.

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