… Reinventing Single Cell Analysis

Expert Session

Amphacademy 2017

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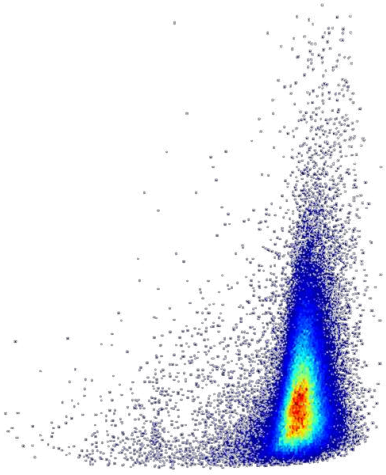
Contents

- From Sample to Result
- Software Features
- Design of Experiment
- Tips and Tricks
- Setup of an Autosampler Measurement



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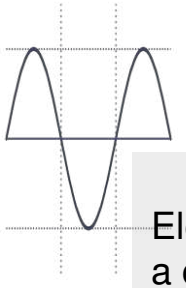
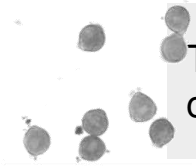
Impedance Flow Cytometry (IFC)



Electrical **Impedance** is opposition to a current in a circuit when an alternating voltage is applied

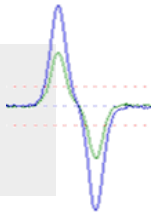
The cells are suspended in a conductive **Fluid**

Impedance Flow Cytometry



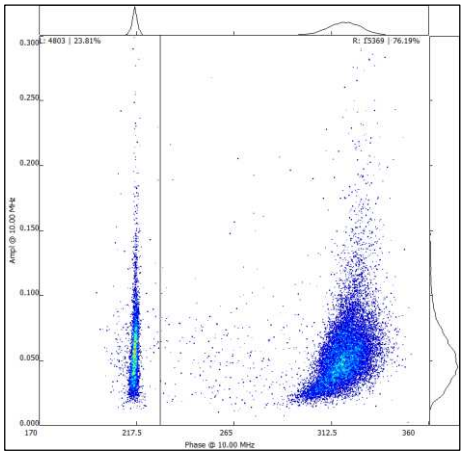
The technology is used to characterize **Cells**

Electrical properties of cells are **Measured**



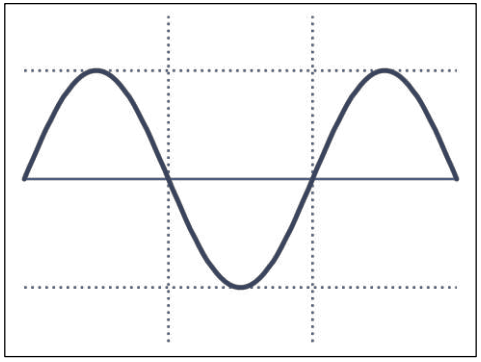
From Sample to Result

?

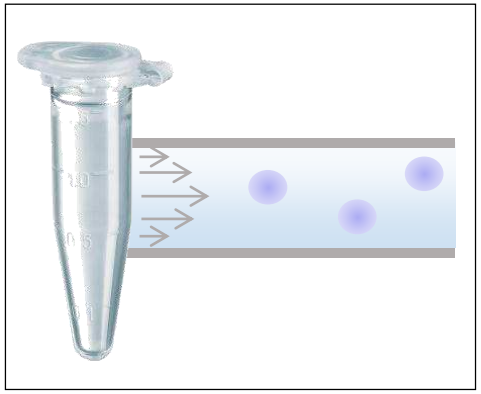


Microfluidic and Microelectronic Technology

Alternating Current Electric Field

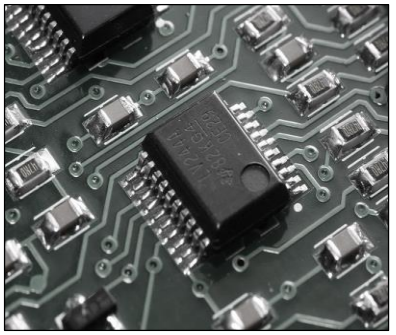


Fluidic System

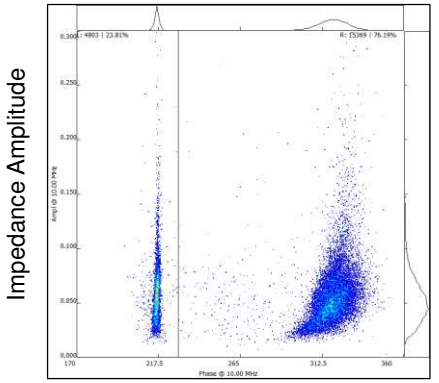


Microfluidic + Microelectronic Chip

Instrument Electronics

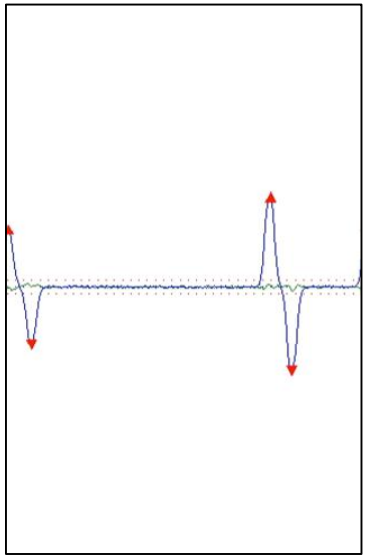


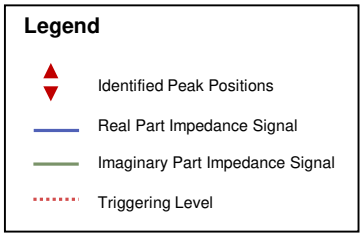
Graphical User Interface



IFC Data Analysis

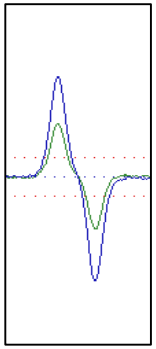
1. Impedance Measurement



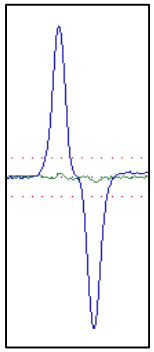


2. Identification of Cells

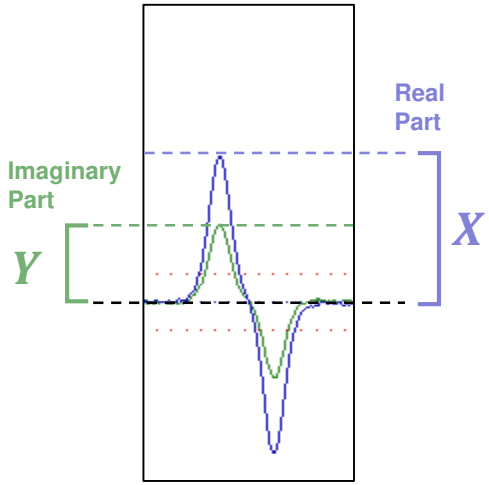
Viable Cell



Dead Cell



3. Extraction of Real and Imaginary Parts



4. Calculation of Impedance Amplitude and Phase

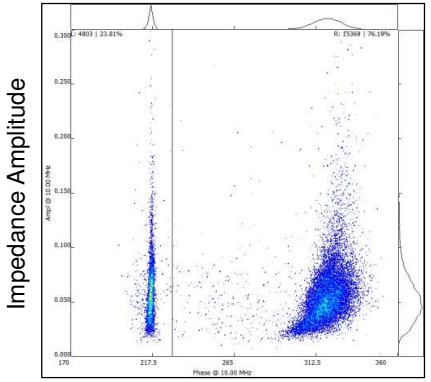
Amplitude

$$A = \sqrt{X^2 + Y^2}$$

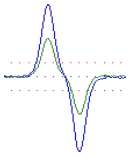
Phase

$$P = arctan\left(\frac{Y}{X}\right)$$

Each measured particle results in a dot in the Phase – Amplitude Scatterplot



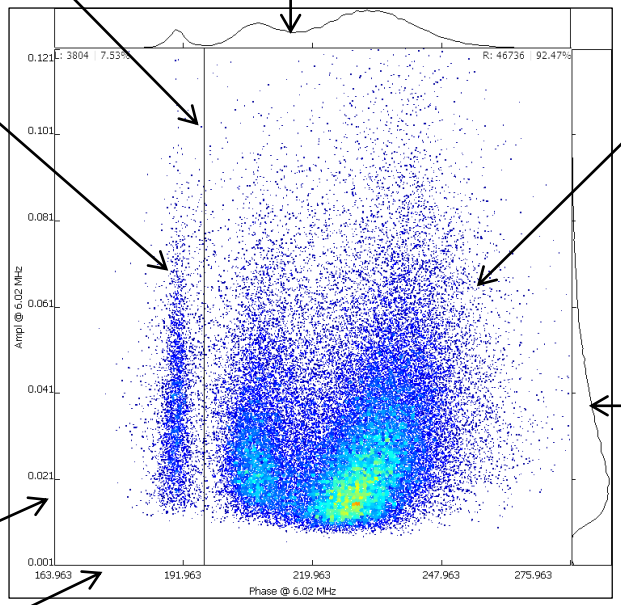
What do we see in a scatterplot?



Each measured particle corresponds to one dot in the density scatterplot

Vertical Gate

Phase Histogram



Impedance Amplitude

Impedance Phase

Density Scatterplot revealing electrically different cell populations

Amplitude Histogram

Amplitude Axis

Phase Axis

Dead cells appear at low phase angles

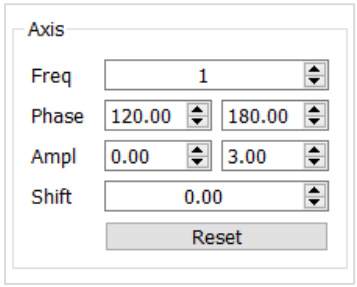
Viable cells appear at higher phase angles

… Reinventing Single Cell Analysis

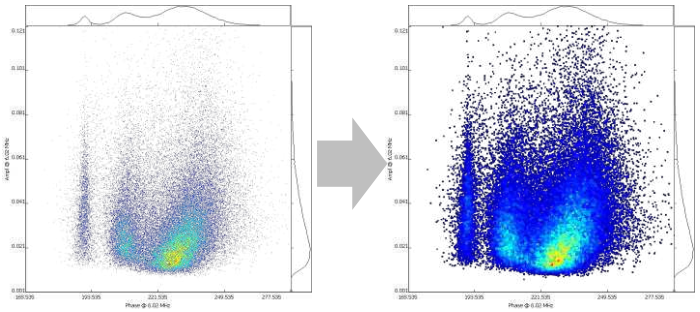
Software Features

Data Analysis – Visualization

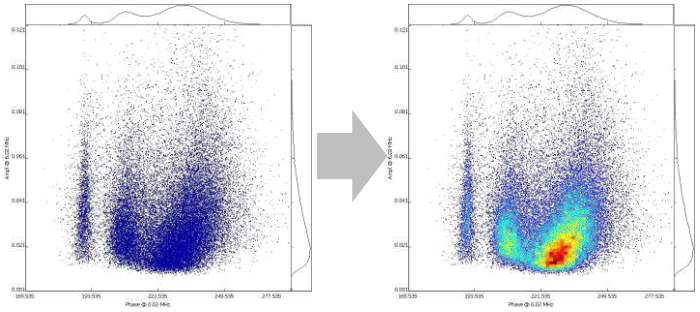
- Changing the position / scaling of the plot
  - Moving: Left-click and drag
  - Zoom: Move mouse wheel in the plot
  - Define axis limits



- Changing the dot size
  - Press *Ctrl* and move the mouse wheel in the plot



- Changing the density plot coloring
  - Press *Alt* and move the mouse wheel in the plot



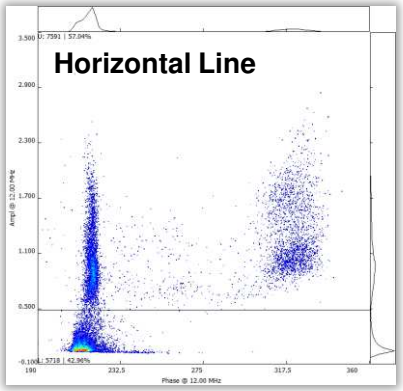
Data Analysis – Gating

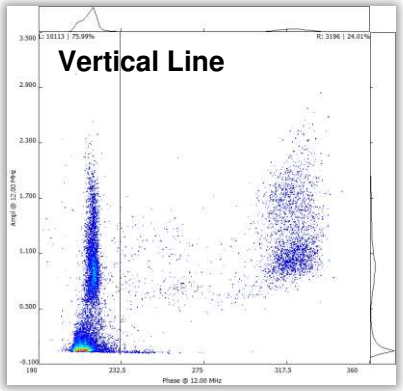
- Horizontal line gate
- Vertical line gate
- Cross gate
- Polygon gate
  - Hide cells
  - Advanced statistics

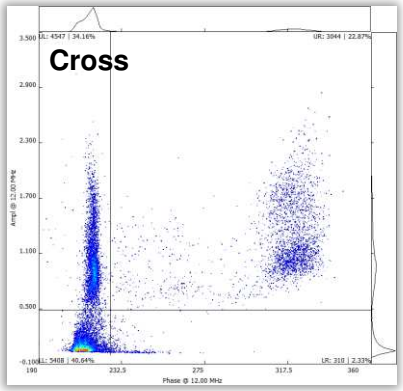
What is the meaning of the gate labels?

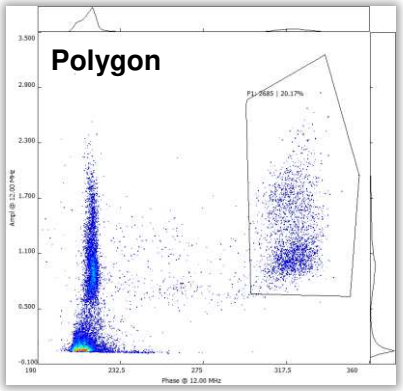
- R: Name of the gate

- 31438: Number of points inside the gate
- 91.18 %: Percentage of points in the gate, with respect to all points in the plot









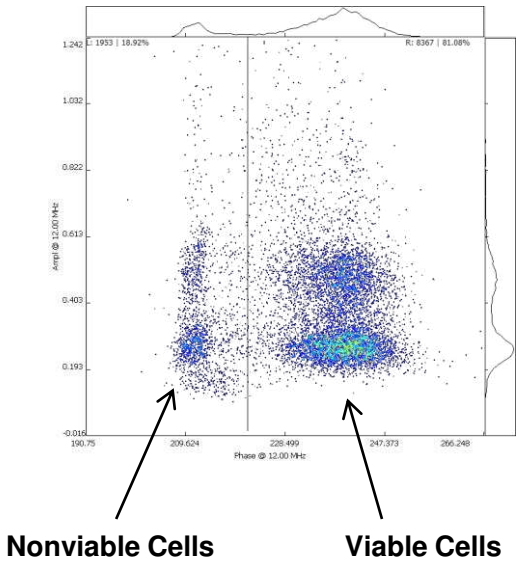
Data Analysis – Gating

- Gating is a process used to quantify different subpopulations of cells. In the case of viability analysis, gates are used to **quantify viable and dead cells**, from which the percentage of viable cells is calculated.

$$V(\%) = \frac{\%viable}{\%viable + \%nonviable} \times 100\,\%$$

- The most important parameter for the viability analysis is the impedance phase (x-axis). Viable cells appear at higher phase angles than dead cells.
- For many samples, a simple vertical line gate is already sufficient to obtain the viability information.

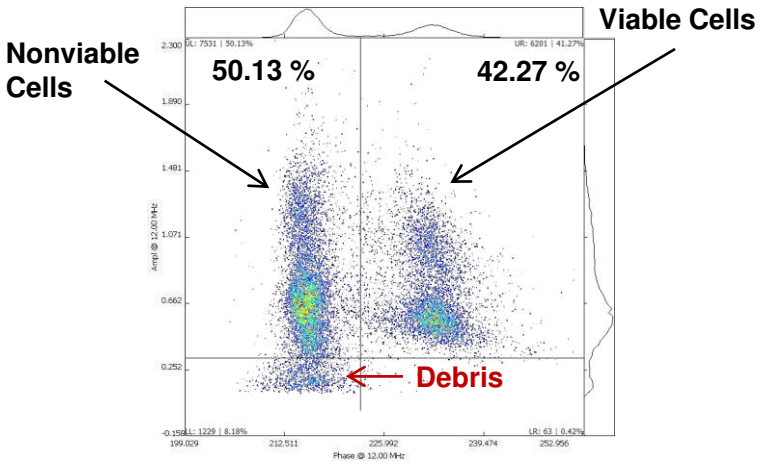
Case 1: No Debris



Data Analysis – Gating with other particles

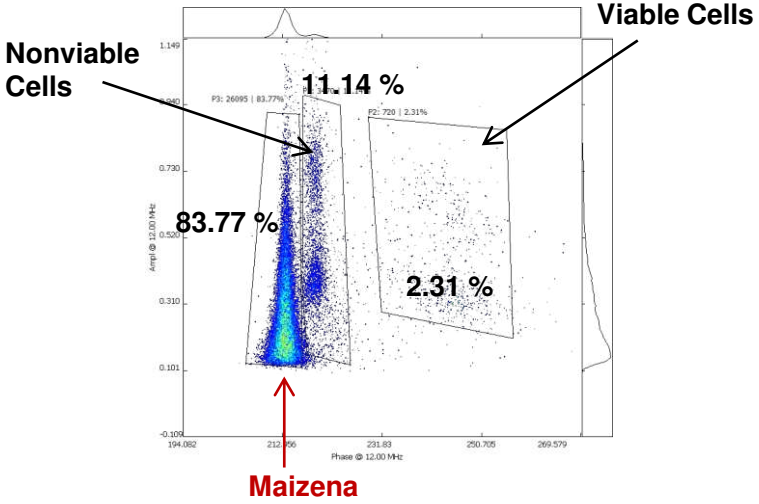
- Depending on the type of sample, other types of particles than pollen cells can be present. In that case, polygon or cross gates are used to correctly quantify viable and nonviable cells.

Case 2: Debris



$$V(\%) = \frac{41.27\ \%}{41.27\ \% + 50.13\%} \times 100\ \% = \mathbf{45.2}\ \%$$

Case 3: Maizena



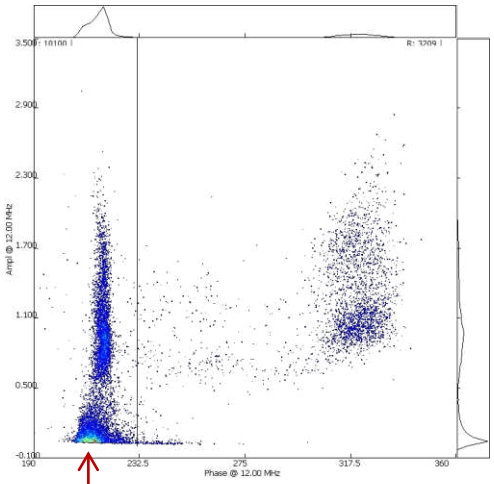
$$V(\%) = \frac{2.31\ \%}{2.31\ \% + 11.14\ \%} \times 100\ \% = \mathbf{17.2}\ \%$$

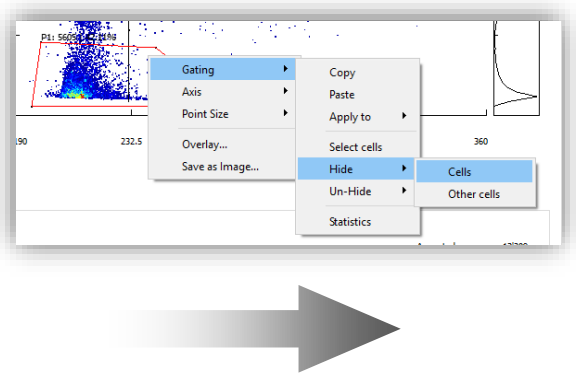
Hide Cells (coming soon)

- *Hide Cells* to exclude debris from the analysis
- Hide *polygon gate* content or everything around the polygon
- Apply *Hide Cells* gate to other measurements as usual

Viability before correction

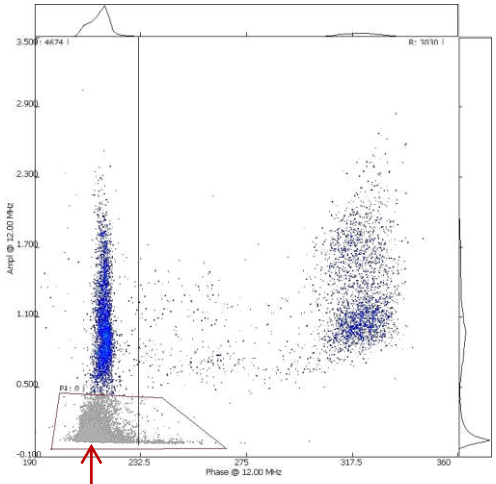
24.1 %





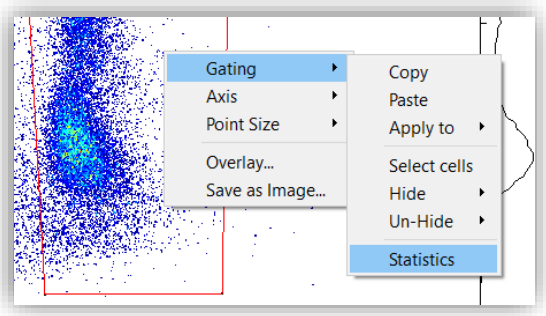
Viability after correction

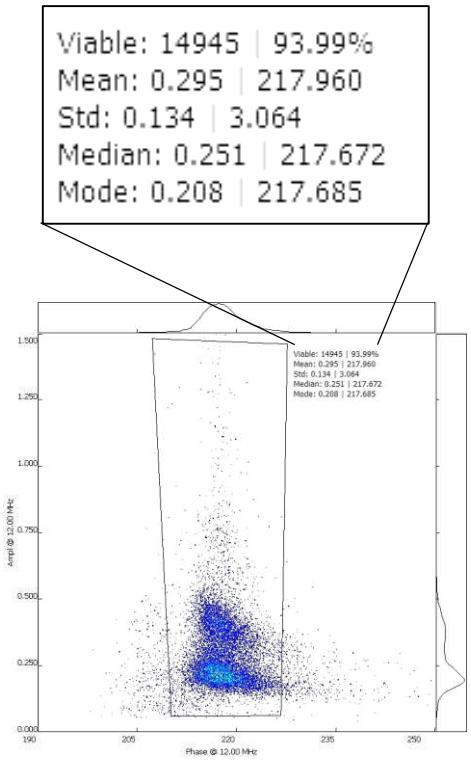
39.4 %

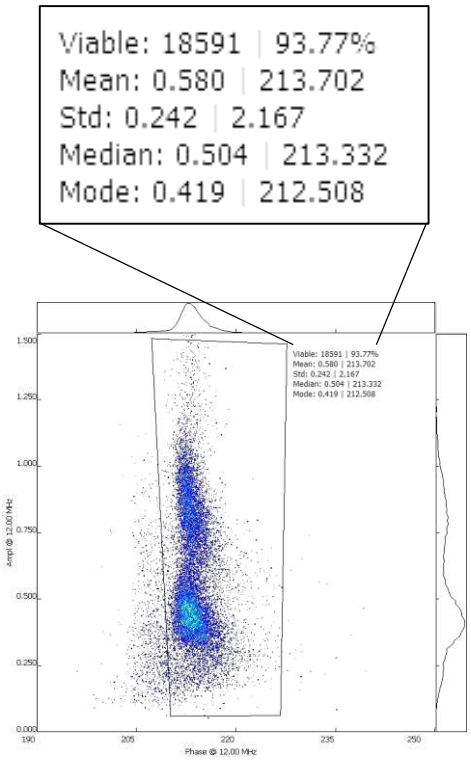


Advanced Gate Statistics (coming soon)

- *Mean*, *Median*, *Standard Deviation* and *Mode* of amplitudes and phases of all points in a *polygon gate*



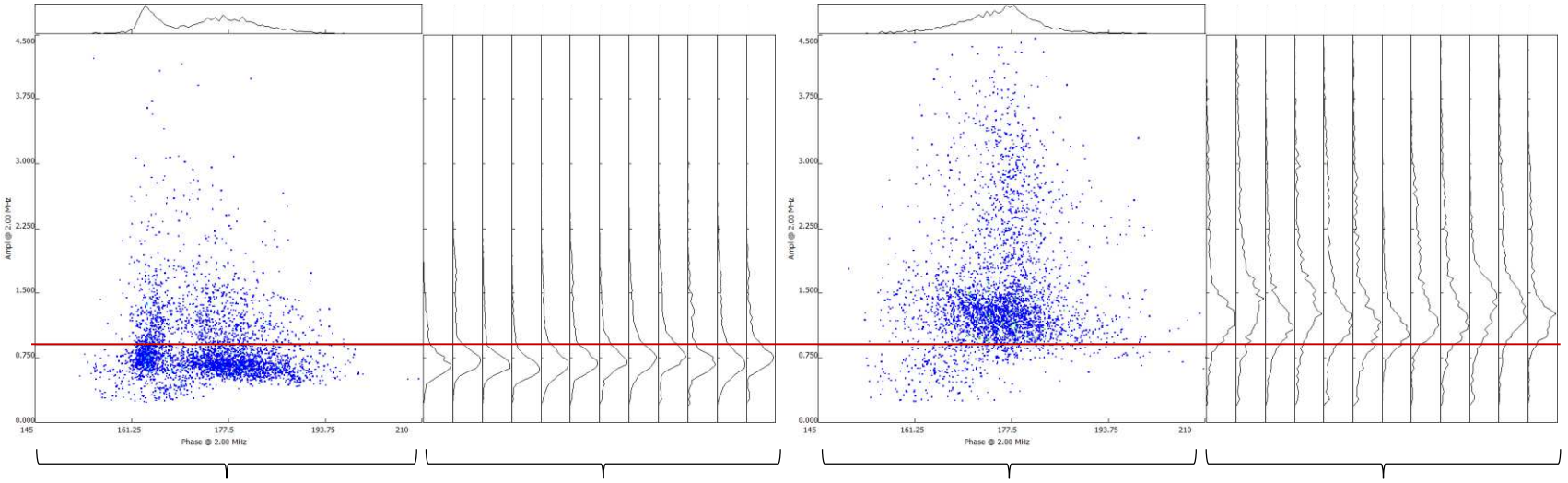




Pollen ploidy based on pollen size differences

2n Genotypes

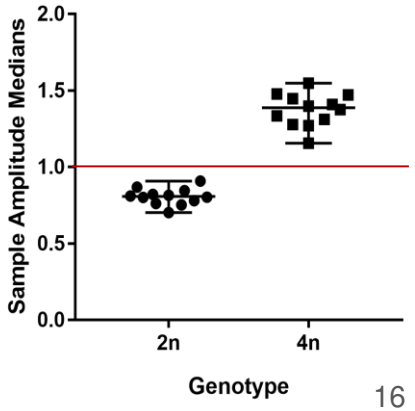
4n Genotypes



2D plots and 1D projections of watermelon pollen after analysis with IFC

- Left stack: Pollen samples of diploid (2n) watermelon genotypes
- Right stack: pollen samples of tetraploid (4n) watermelon genotypes

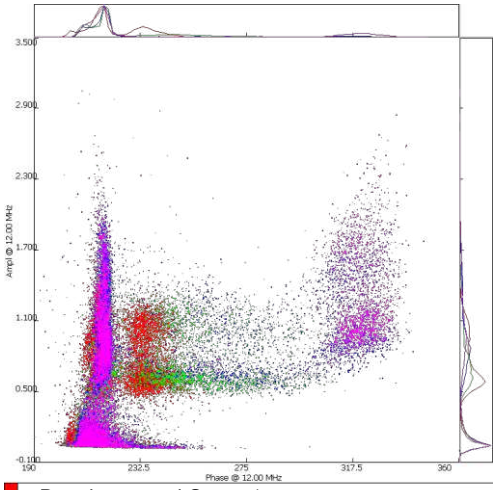
Median Amplitudes for 2n and 4n genotypes →



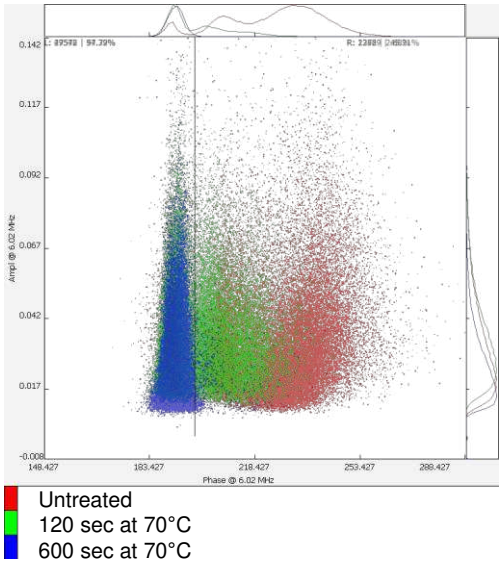
Plot Overlay (coming soon)

- Visualize processes or differences (e.g. Maturation, heat treatment, ploidy)
- Use the same axis settings for all plots to overlay, then highlight the desired measurements > right-click in plot > *Overlay*

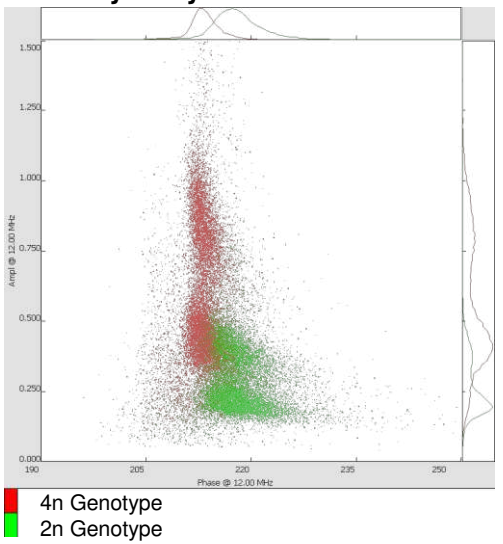
Maturation of Wheat Pollen



Heat - Treatment of Yeast

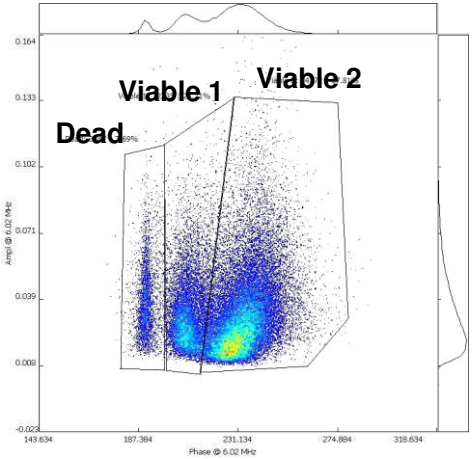


Ploidy of Cyclamen Pollen

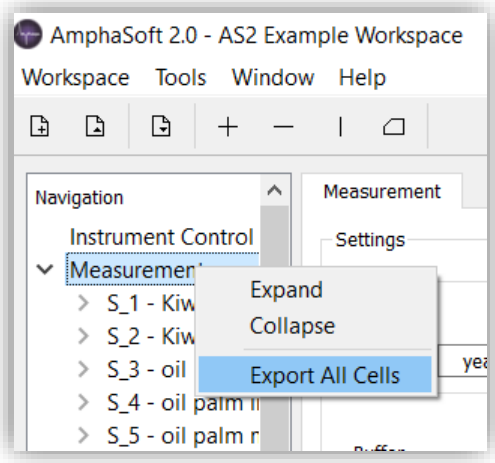


Data Export (coming soon)

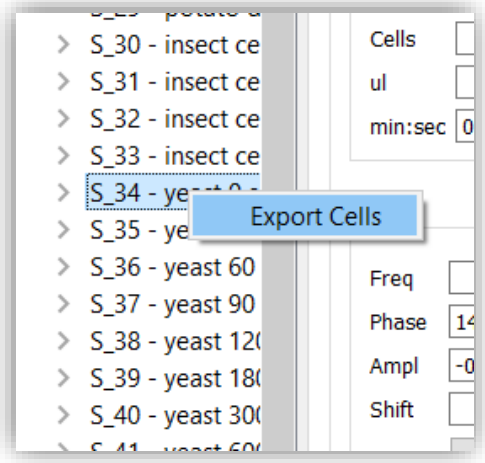
- For advanced data analysis with custom algorithms
- Phase, amplitude and corresponding gate of each particle in *.csv* format
- Export of all data
  - Right-click on Measurements > *Export All Cells*
- Export of single-measurement data
  - Right-click on measurement > *Export Cells*



Export of all data



Export of single measurement data

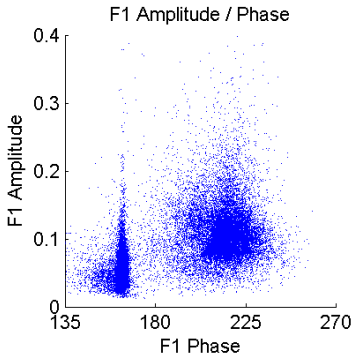


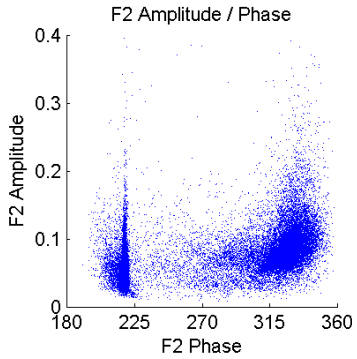
Phase / Amplitude / Gate Data

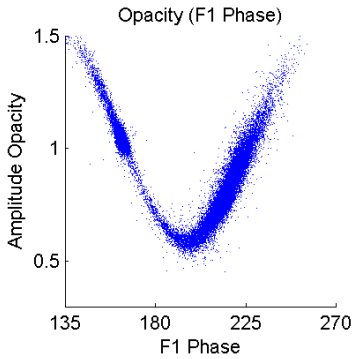
	A	B	C
1	247.485	0.059732	Viable 2
2	235.866	0.078129	Viable 2
3	221.102	0.07715	Viable 1
4	210.053	0.031917	Viable 1
5	206.661	0.024568	Viable 1
6	238.14	0.053583	Viable 2
7	189.229	0.071557	Dead
8	231.657	0.022509	Viable 2
9	243.51	0.030326	Viable 2
10	223.015	0.049962	Viable 2
11	253.639	0.050914	Viable 2

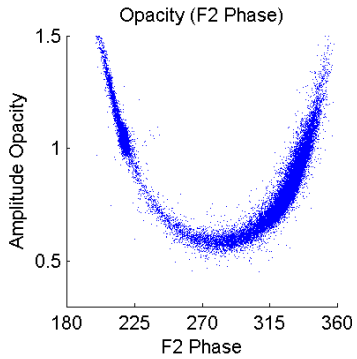
Example of offline data analysis

5 µg/ml Amphotericin B

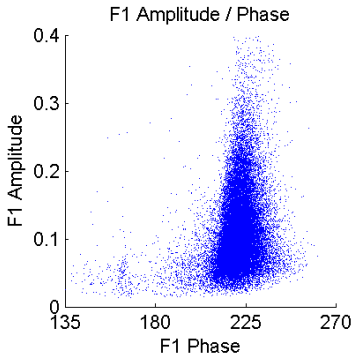


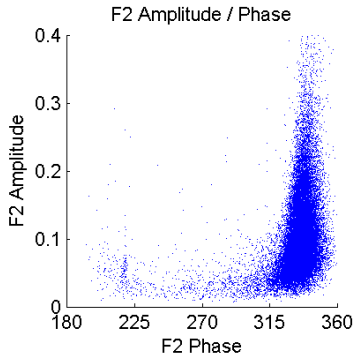


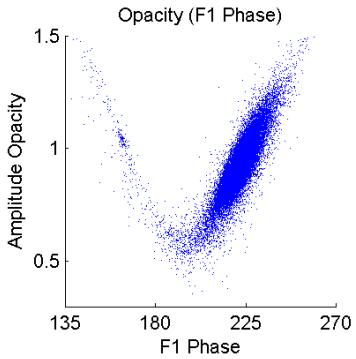


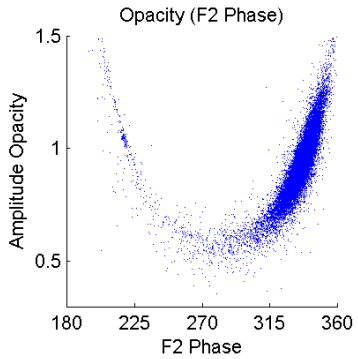


Control









Play

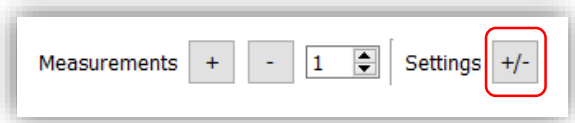
Yeast cultures, 120 min time lapse

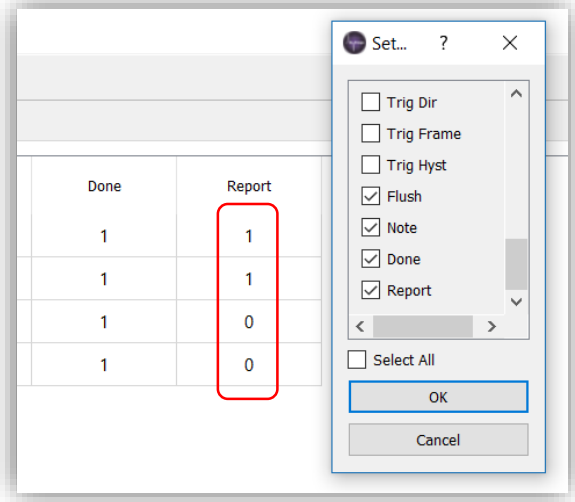
Reporting and Plot Export

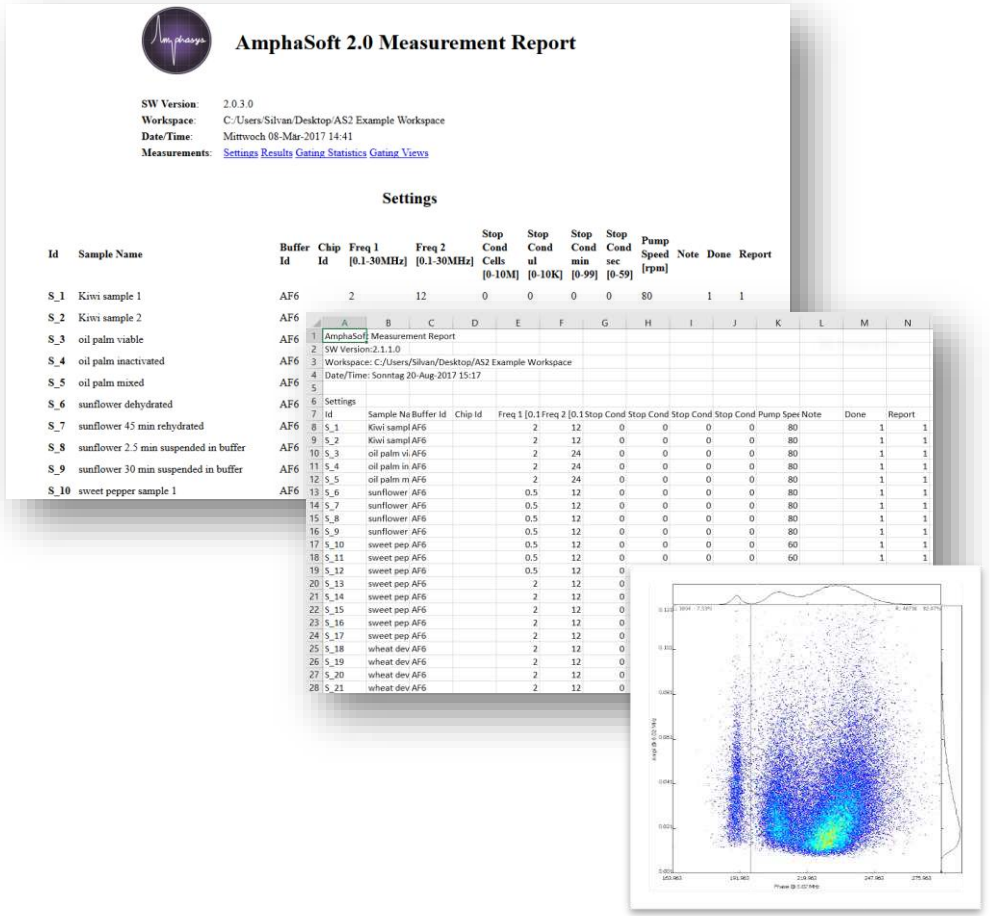
- *.csv* report
- *.html* report

Select which measurements you want in the report!

- Plot export



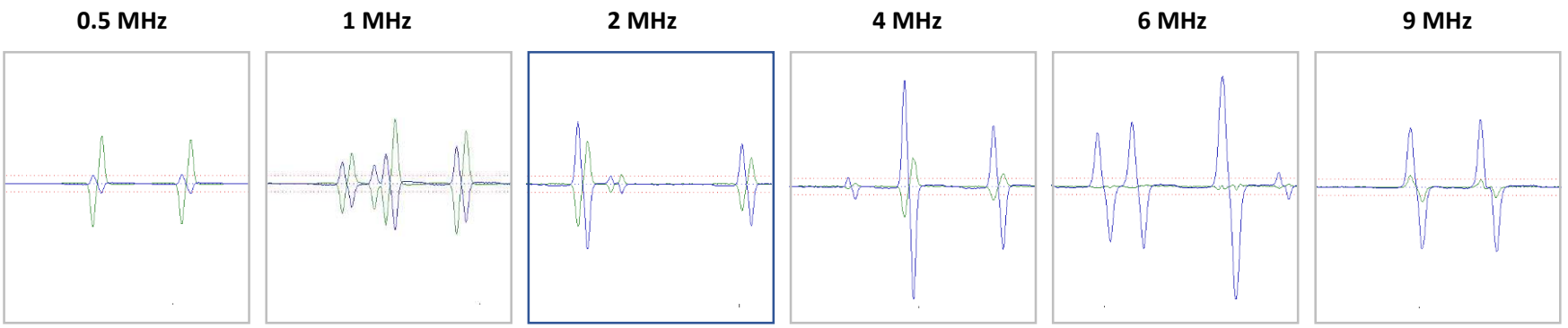




Manual Instrument Controls

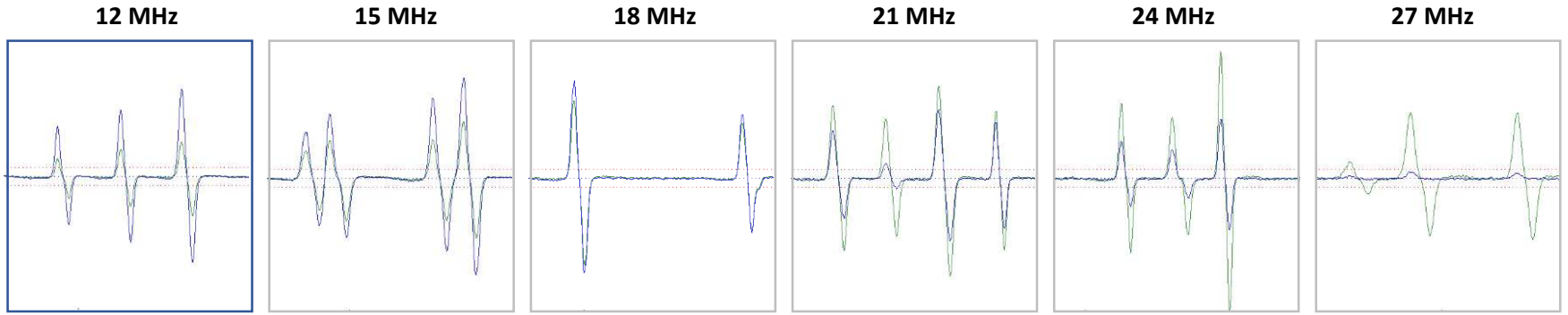
Advanced Tab

Triggering Source and Direction



Source: **Y** Direction: **Negative**

Source: **X** Direction: **Positive**



Source: **X** Direction: **Positive**

Source: **Y** Direction: **Positive**

Real Part Impedance Signal (X)

Imaginary Part Impedance Signal (Y)

Signal properties depend on frequency, chip type and quality, electronic gain, buffer and cell type!

Design of Experiment

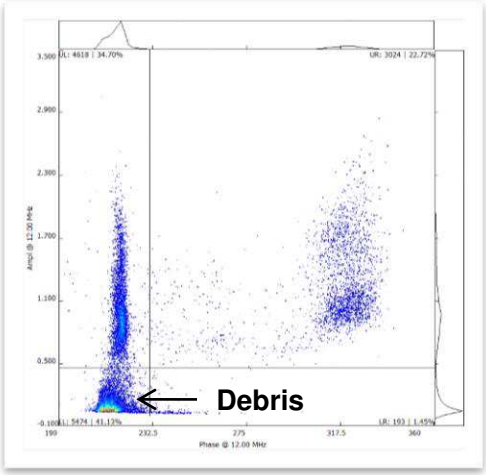
Design of Experiments

- What cells I want to measure?
  - **Cell type** determines **Buffer**
  - **Cell size** determines
    - **Filter size**
    - **Measurement Chip**
    - **Instrument Settings**
  - Always consult the pollen list!

- What parameter do I want to determine?
  - Viability, concentration, ploidy, developmental stages...Design your experiment!
  - Prevent sampling bias
  - Prevent measurement and data analysis bias

Preventing Experimental Bias

- Do not compare apples with oranges
  - Variability within a plant, flower, tassel...
  - Variability during the day
  - Different developmental stages
  - Environmental factors
- Know the stability of the cells in the buffer
- Properly rehydrate / equilibrate after freeze-storage
- Work with standardized protocols
  - Pollen source
  - Buffer
  - Chip Type
  - Settings
  - Filter Size
- Account for debris in the analysis of data
  - Mathematically
  - *Hide Cells* feature



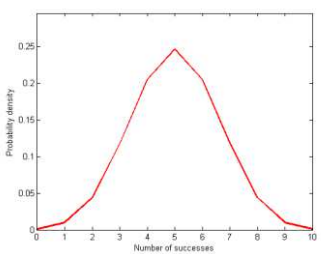
Viability – How many cells to measure?

Viability – Binomial Distribution

Viability = 50 %

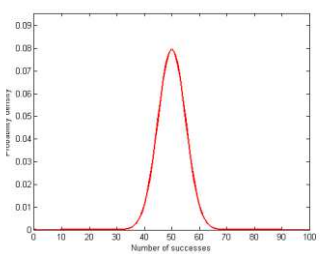
(P_{model} = 0.5)

N = 10 cells



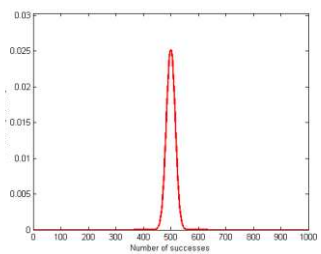
95 % CI
[0.190 – 0.810]

N = 100 cells



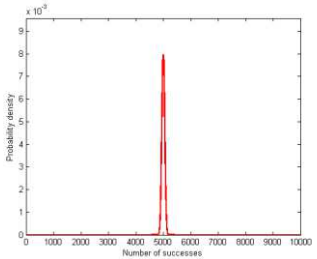
95 % CI
[0.402 – 0.598]

N = 1’000 cells



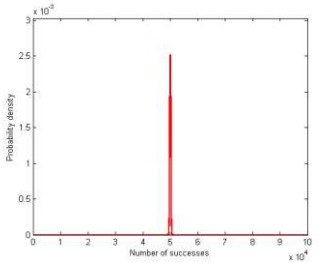
95 % CI
[0.469 – 0.531]

N = 10’000 cells



95 % CI
[0.490 – 0.510]

N = 100’000 cells



95 % CI
[0.497 – 0.503]

Probability Density

$$\Pr(X = k) = \binom{n}{k} p^k (1-p)^{n-k}$$

$$\binom{n}{k} = \frac{n!}{k!(n-k)!}$$

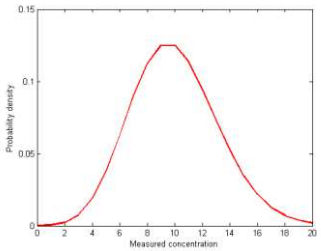
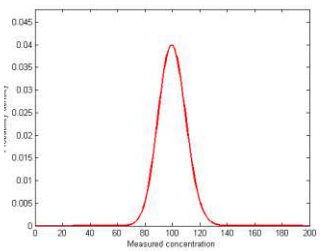
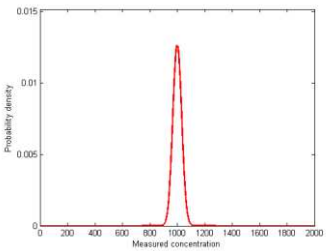
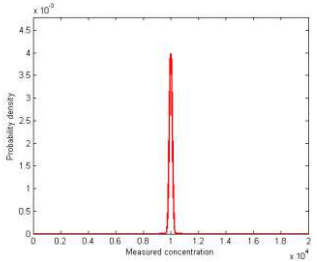
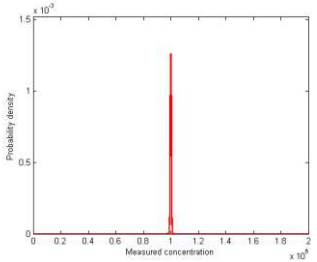
Confidence Interval

$$\hat{p} \pm z\sqrt{\frac{\hat{p}(1-\hat{p})}{n}}$$

- The model predicts that measurements with **> 10’000** acquired cells give very precise results
- Gating is easier for a large number of measured cells
- For rough estimates, 500-1’000 cells can already be sufficient

Concentration – Precision

Concentration – Poisson Distribution

C = 10 cells/ml	C = 100 cells/ml	C = 1’000 cells/ml	C = 10’000 cells/ml	C = 100’000 cells/ml
				
95 % CI [5 - 18]	**95 % CI [81 - 121]**	**95 % CI [938 – 1’064]**	**95 % CI [9’805 – 10’198]**	**95 % CI [99’381 – 100’622]**

Probability Density

$$P(k \text{ events in interval}) = e^{-\lambda}\frac{\lambda^k}{k!}$$

Confidence Interval

$$\tfrac{1}{2}\chi^2(\alpha/2;2k) \le \mu \le \tfrac{1}{2}\chi^2(1-\alpha/2;2k+2),$$

- The model predicts that measurements with **> 10’000** cells/ml give very precise concentration results
- For rough estimates, 100-1000 cells can already be sufficient
- Accuracy depends on the cleanliness of the flow sensor

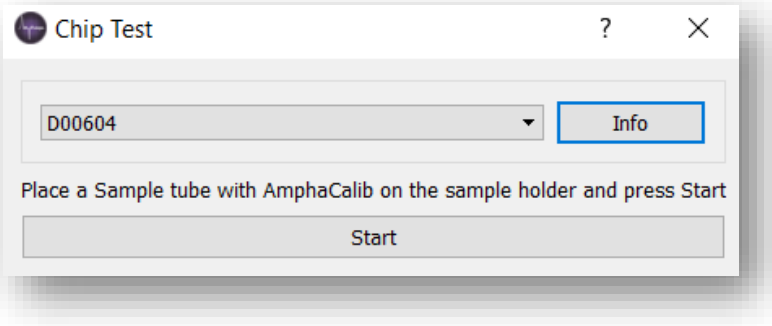
Model does not take particle sedimentation into account

Tips and Tricks

Tips and Tricks

Chip Test

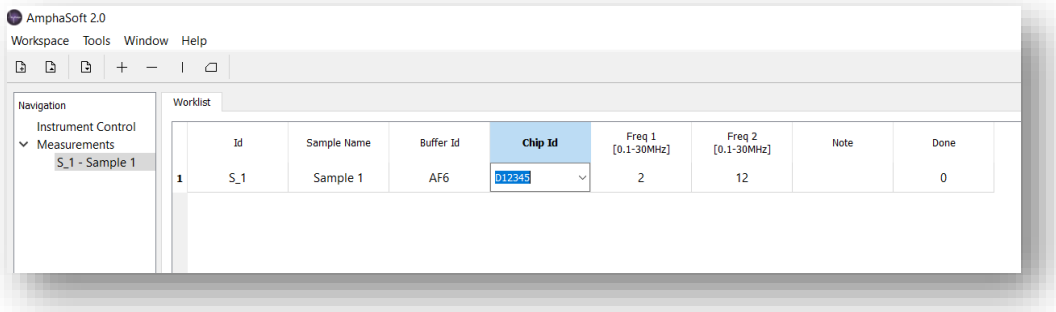
- A Chip Test is a procedure to evaluate the quality of a measurement chip
- Perform Chip Tests on a regular basis to ensure a good quality of the measurements
- Launch a Chip Test in the Menu under *Tools* > *Chip Test*. The Chip Test requires 2 ml AmphaCalib buffer.
- The *Info* button reveals the number of measurements performed with a Chip



Tips and Tricks

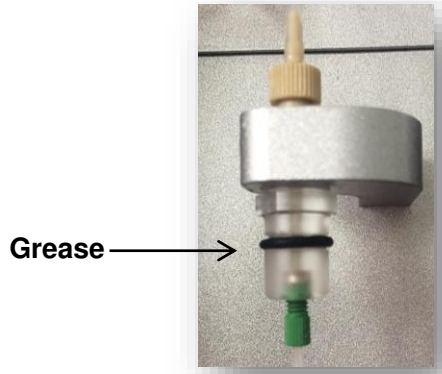
Worklist

- Always confirm new configurations (Sample name, Chip name, Buffer ID, Stop Conditions...) with *Enter*



O-ring at the Sample Holder

- Use a bit of grease if it is hard to attach tubes



Batteries

- Fully charge once per month to ensure durability

Tips and Tricks

Sample Preparation

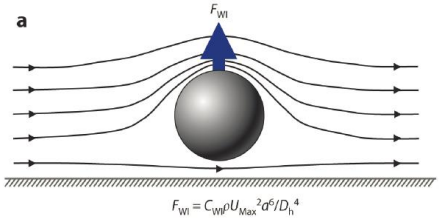
- Use a standardized sampling method
- Use a filter with a mesh size of 1.5 – 2 times the diameter of the cells
- Try to minimize the amount of debris
  - If pollen is extracted from anthers, squeeze the anthers gently with a pistil
- Hydrophobic pollen: Add 0.05 % Tween20 (detergent)
- Seal sample tube with Parafilm and quickly invert before measuring
  - Distributes sedimented particles
  - Reduces the chance of clogging
  - Improves concentration accuracy

Measurement

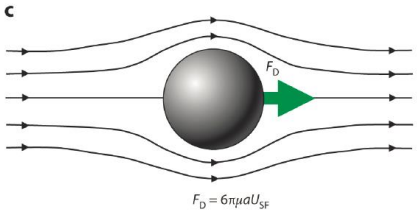
- Use a chip with a channel of about 2 – 3 times the size of the pollen

Inertial Focusing – Data Interpretation

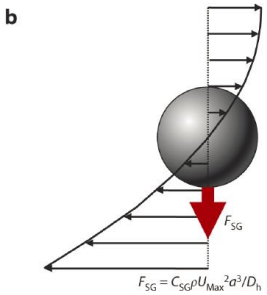
Wall-induced lift force



Viscous drag force



Shear-induced lift force



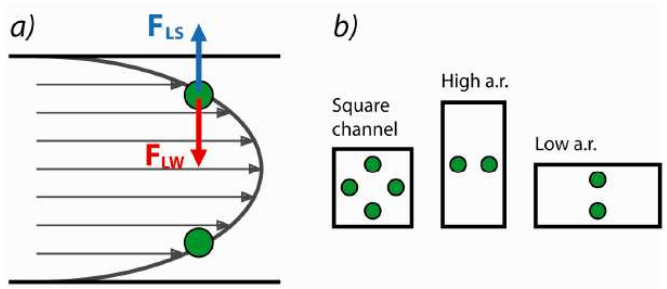
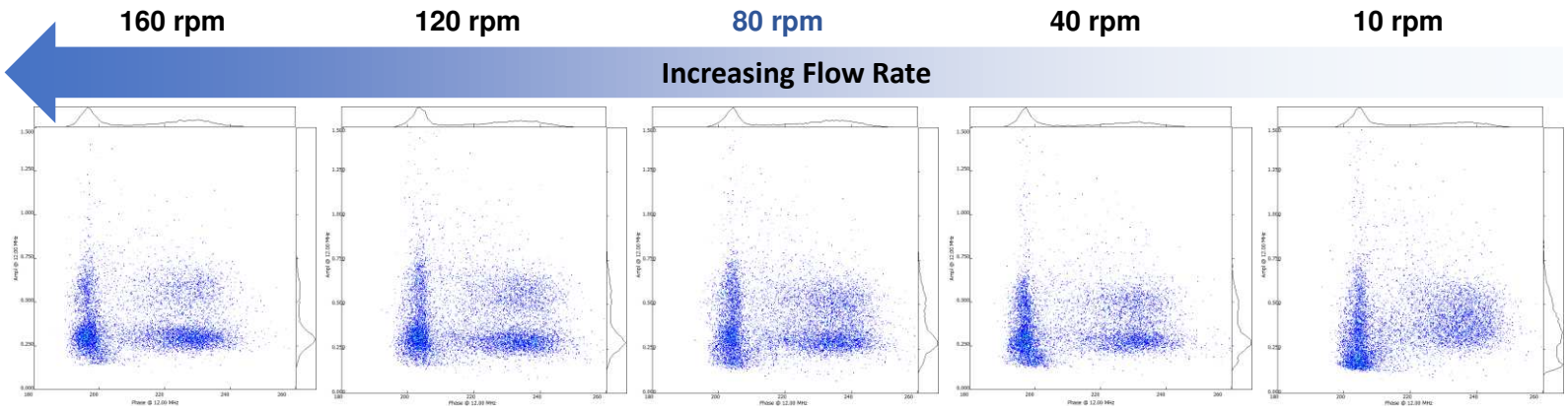


Figure 1: Particle inertial focusing in flow through straight channels. (a) The shear induced lift force (F_{LS}) and a wall induced lift force (F_{LW}) acting on a particle flowing in a microchannel. (b) Illustration of the cross-sectional equilibrium positions of particles flowing through different channel geometries.



… Reinventing Single Cell Analysis

Autosampler Measurements

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