University of Twente

Bachelor Thesis Electrical Engineering

Optimizing differential impedance flow cytometry to assess the calcification state of calcifying algae

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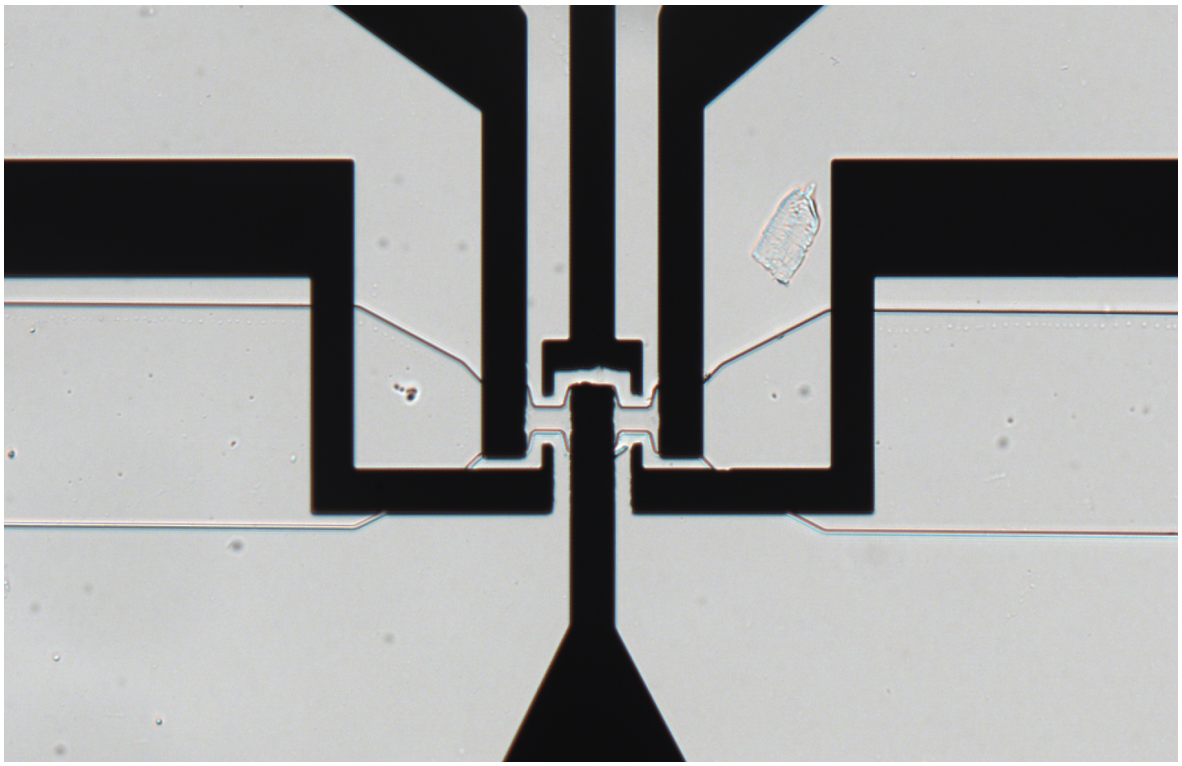

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Chapter 1

Introduction

Ocean acidification caused by industrial carbon emissions cause a threat to calcifying algae which are an important part of the ocean carbon cycle [2]. The analysis of the calcification state of a population of coccolithophores can give more insight into the effects of ocean acidification on the studied species coccolithophore Emiliania huxleyi. These cells play an important part in the oceans carbon cycle by absorbing carbon from the ocean using photosynthesis and calcification, this carbon is then stored once the cell sink to the ocean floor after their death. The role of these cells in the ocean carbon cycle is why analysis of this species' response to ocean acidification by an increasing concentration of carbon dioxide (CO2) in the atmosphere is worth researching [3].
The coccolithophores use CO2 to create organic carbon using photosynthesis which is stored inside the cell. These calcifying algae also create inorganic calcium carbonate (CaCO3) platelets called coccoliths by converting two bicarbonate ions into the CaCO3, this reaction also reintroduces CO2 into the ocean [3]. These platelets form an exoskeleton surrounding the cell. The presence of the platelets influences the ratio of particulate organic carbon (POC) and particulate inorganic carbon (PIC), this is called the PIC:POC ratio. As the coccoliths contain the inorganic CaCO3, a higher PIC:POC ratio corresponds to a larger presence of coccoliths. The carbon cycle of the coccolithophores and ocean is also shown in figure 2.1.
For a more detailed analysis of populations of the coccolithophores, a technique called impedance flow cytometry is used where individual cells pass a pair of electrodes on a glass plate that can be used to measure the impedance between the electrodes [8]. To guide the coccolithophore cells to the correct position a polydimethylsiloxane (PDMS) chip containing channels is added on top of the glass-electrode plate, together this forms a glass-PDMS chip for single cell impedance measurements.
This research will focus on using this impedance flow cytometry system to analyse the influence of coccoliths on the impedance of the entire cell, this can be done by looking at the difference of populations of coccolithophores with and without coccoliths one the cell, whether or not coccoliths are present on the cell is called the calcification state. Different parameters in the system can be altered in the system to optimize the difference in responses between the two coccolithophore groups. This could later be used in further research to classify the amount of coccoliths present on an unknown cell and with this the PIC:POC ratio of a cell.

1.1 Research question

Does the introduction of differential measurements allow for reliable classifications of the calcification state of calcifying algae?

1.2 Sub questions

In addition to the main question there are sub questions that need to be addressed to come to a good discussion to answer the main question. These questions are placed in chronological order and build on the answers to the previous subquestions.

- What are the differences in the measurements of signals generated with differential and previous non-differential systems?
- How can the data of the differential measurements be converted to a measure of calcification state?
- What is the influence of different measurement parameters like voltage and frequency on the reliability and accuracy of the measurements?
- Do the differential measurements give a more reliable method for the classification of the calcification state of calcifying algae compared to previous measurement systems?
- Does the new system allow for estimating the PIC:POC ratio of a single cell?

Chapter 2

Theory

2.1 Coccolithophores

This research focuses on measuring and using the impedance of individual coccolithophore cells, looking at the Emiliania huxleyi species in particular. The production of a calcium carbonate exoskeleton changes the size and impedance of the cell. When the coccolithophores die they sink to the bottom of the ocean, causing an outgoing flux of carbon from the seawater. The structure of the coccolithophore cells is also shown in figure 2.1.

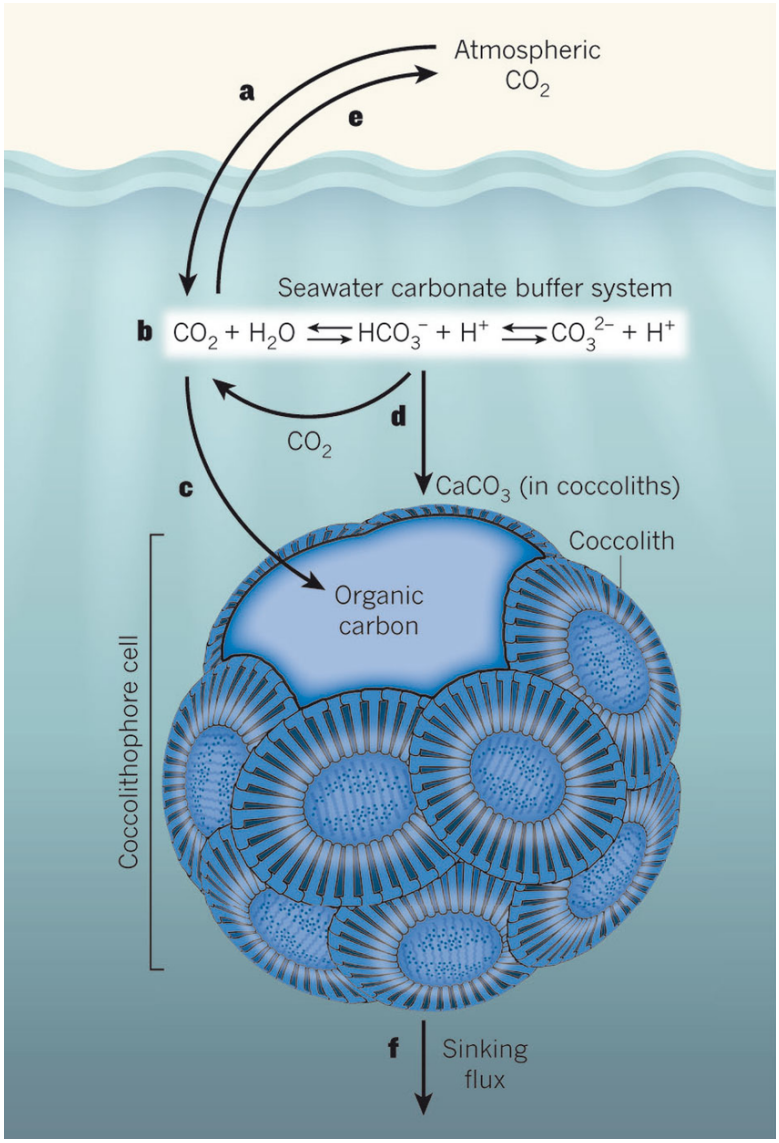

Figure 2.1: *Coccolithophores and their carbon cycle [3]*

2.1.1 Decalcification

The coccoliths of a population of coccolithophores can be dissolved in an acidic environment and a cell that is completely stripped of its calcium carbonate platelets will be called a decalcified cell. In nature decalcification occurs through acidification of the ocean by the conversion of carbon dioxide into carbonic acid. In the lab this can be simulated by adding carbon dioxide (CO2) to a population of these cells in a seawater medium, in this research the coccoliths are removed completely to maximize the difference between normal cells and decalcified ones. The cells survive this process of decalcification and will start to regrow coccoliths once the medium becomes less acidic. This happens over time as the carbonic acid is converted to gaseous carbon dioxide again. Figure 2.2 shows the two calcification states used in this research, where one of the populations has a healthy amount of coccoliths and the other was treated with CO2 to dissolve the platelets completely. The calcified coccolithophore cells have a size of 4 to 5 µm and the decalcified cells are smaller after the removal of the platelets.

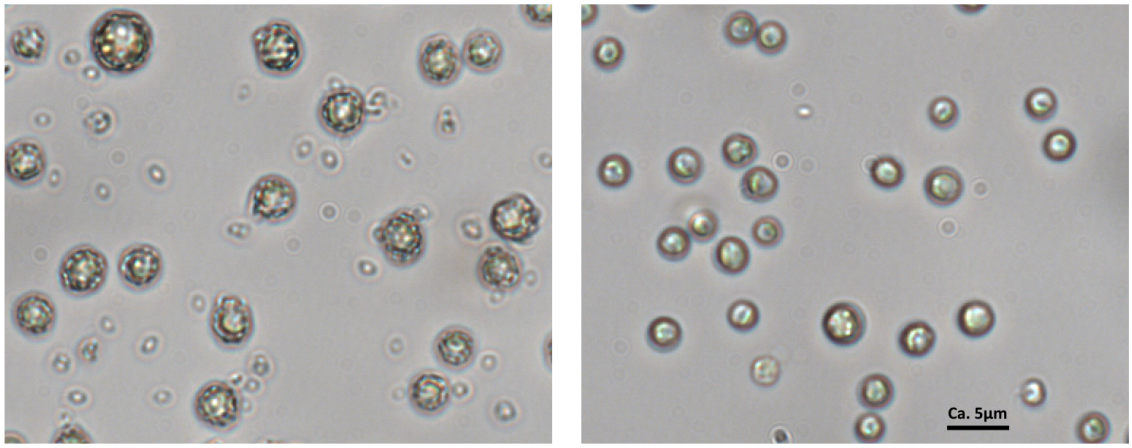

Figure 2.2: *Normal calcified cells (left) and decalcified cells (right)*

2.2 Impedance spectroscopy

Coccolithophores will be analysed by passing single cells past a pair of electrodes on which a known voltage is applied. Between the electrodes a current will flow between the electrodes, when no cells are present the current will be determined by the impedance of the seawater medium, once a cell passes between the electrodes the impedance will change and so will the current. The current can be converted back into a measurable voltage with a transimpedance amplifier.
By comparing the impedance of both calcified and decalcified cells at just one frequency it is possible to get an estimate for the size of the cell, however, as small cells with coccoliths can have a similar impedance to larger cells without coccoliths this alone does not give sufficient information about the existence of these platelets. By doing impedance measurements at different frequencies the impedance spectrum is sampled, if one of the frequencies is used to normalize the measured cells for size other frequencies can be used to detect differences in the spectrum.

2.2.1 Lock-in amplifier

A lock-in amplifier is used to generate the voltage applied on the electrodes and to measure the returning signal. A lock-in amplifier generates high frequency signals between 0 Hz and 50 MHz and uses demodulation on the same frequency to return a baseband signal proportional to the magnitude of the returning high frequency signal.
Using this method, multiple frequencies can be applied to the electrodes at the same time. A different demodulator is used for each frequency and a low-pass filter is then applied to the different signals to filter out the influence of other frequencies.

2.2.2 Complex nature of impedance

Per definition an impedance has both a real and imaginary part, different resistive parts of the cells will correspond to a real impedance but the system also contains a couple of capacitive elements that can be measured as the imaginary part of the impedance.
This complex signal can either be expressed with a magnitude and phase or with a real and imaginary component. The Lock-in amplifier is able to measure both these variants by using two voltage measurements. The real component of the signal is retrieved by demodulating the signal directly, this creates an in-phase signal. The complex part is measured by applying a 90 degree phase shift to the oscillator signal before demodulating, this now outputs a quadrature signal.

When required these two measurements can be transformed into a magnitude and phase signal, these two systems are connected as shown in figure 2.3.

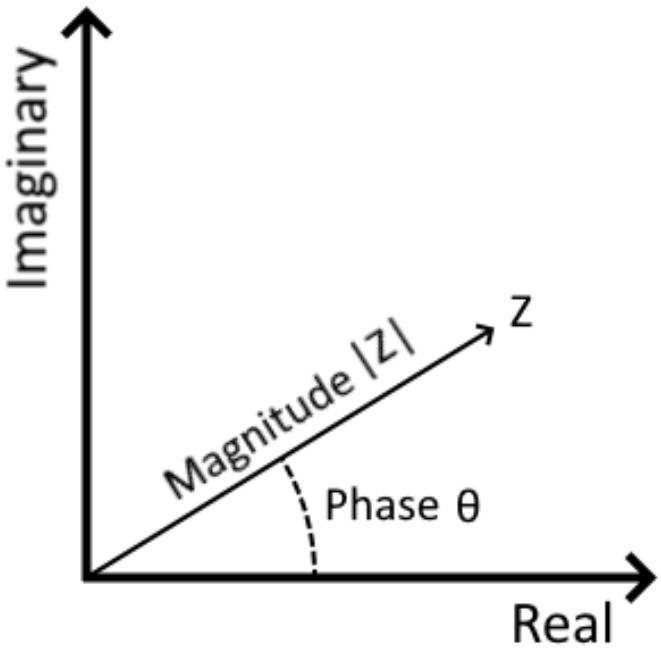

Figure 2.3: *Diagram on the relation between the Real and Imaginary parts of the impedance and the Magnitude and Phase of that impedance*

2.3 Preceding work

This research is part of a larger project by Douwe de Bruijn. Previously measurements have been done using a similar setup, here one pair of electrodes was used as can be seen in figure 2.4. The generated signal was a measure of the total impedance between the electrodes, this consists of the impedance of both the medium and passing cells and was expressed in total magnitude and phase of the impedance [2].

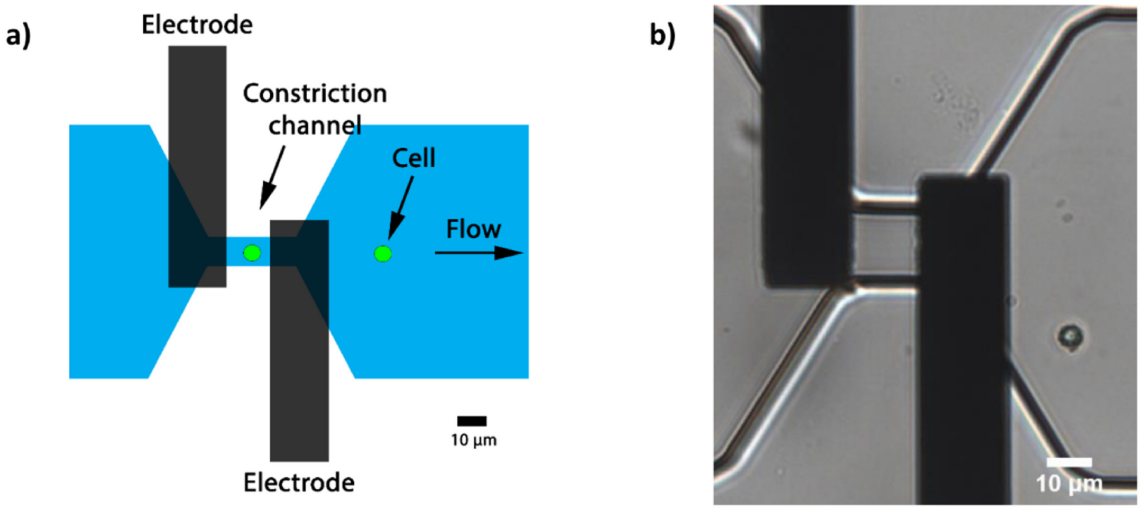

Figure 2.4: *Top view of the previous measurement setup. Diagram (a) and image (b) of the electrodes and fluid channels [2].*

Although the previous setup did provide useful results the setup could still be improved, this research will test a new setup and will analyse how different adjustments to the setup affect the result .

2.4 Differential measurements

The largest adjustment in the new setup is the use of a third electrode, this allows differential measurements to be done. Next to this, a set of ground electrodes have been placed around the other three electrodes to reduce the parasitic capacitance and external interference in order to reduce the noise.
With the addition of a third electrode, one pair will measure the impedance of a passing cell and medium while the other only measures the impedance of the medium, by subtracting one of the signals from the other the effect of the medium will mostly be removed, it also allows the removal of shared noise. This change also means that the resulting signal is no longer a direct measurement of impedance as the difference in current is measured instead of the total current. The rest of this research will now focus on the height of generated peaks of the measured real and quadrature signals instead of the absolute impedance.

2.4.1 Electrodes and channels

To measure the response of a single passing cell it is important to get the cells into a know position, the fluid channels are made $9\mu m$ high to allow the particles to pass through cleanly but to constrict them to a close distance to the electrodes. The electrodes are placed $20\mu m$ apart which is about 4 times as wide as the width of a cell, this allows the particles the be measured for a sufficient time. Figure 2.5 shows a schematic side view of the electrodes and channel.

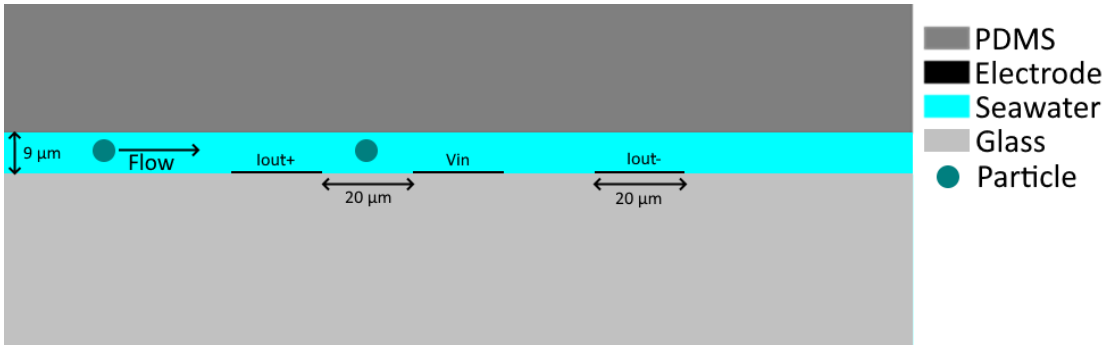

Figure 2.5: *Side view of electrodes and channel*

2.4.2 Expected signals

A passing cell or other particle should generate two opposite peaks in the signal, one for each electrode pair. As the distance between the electrodes is $20\mu m$ and the measured particles should not exceed $5\mu m$ in size, a peak should consist of three parts: a rising edge as the particle passes the first electrode, a constant part where the entire particle is in between the electrodes and a falling edge when the particle passes over the second electrode. Once the particle passed the second electrode a second peak of similar shape but of opposite sign will be generated. This expected signal is visualized in figure 2.6. The most interesting part of this signal is the peak height A, to further reduce possible errors in finding this value both the positive and negative peaks of the signal are measured and averaged. In tests each frequency channel used will generate its own sets of peaks with different amplitudes but the width and shape of these peaks will be similar.

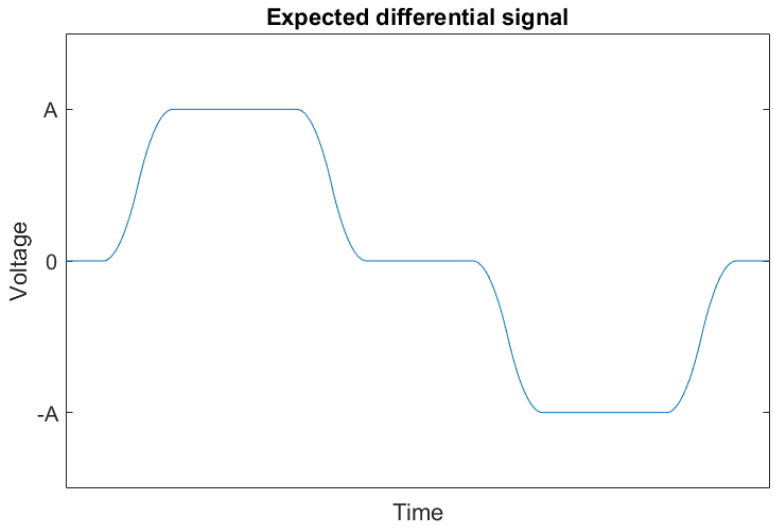

Figure 2.6: *Example of the signal generated by a passing cell*

Complex signals

As both the in-phase and quadrature signals measured by the lock-in amplifier contain useful information it is important to analyse both signals. The resulting signal will now contain two sets of the peaks shown in figure 2.6.

Bandwidth and flow rate

The measurement setup has a list of parameters that influence the shape, width and height of peaks generated by passing cells, a full list of parameters will be provided in the method but here parameters that influence the width of a signal peak are already considered.
Two parameters are worth considering for the width of a peak, these are the flow rate of the fluid and the bandwidth of the low-pass filters in the lock-in amplifier.
As with all filters, the low-pass filters in the lock-in amplifier have a settling time. This is the time required for the output of the filter to rise to a certain level when the input signal changes. The velocity of a passing cell puts a lower limit on the bandwidth of the low-pass filters, however, a larger low-pass filter bandwidth also allows more noise to pass the filter. The low-pass filter bandwidth setting corresponds to the -3dB frequency of the filter. The signal would need to consist of lower frequencies than this bandwidth but as the shape of the peaks won't resemble a sine wave it is hard to find the maximum frequency present in the signal. To guarantee a proper measurement of the signal, this setting is left to set experimentally.
The flow rate of the fluid through the channel influences the time a particle spends between two electrodes, a higher flow rate decreases the time a particle can be measured but it increases the total particle throughput over time, if the settling time of the lock-in amplifier low-pass filter is small enough and the sampling rate is sufficient than the flow rate can be increased to increase the particle throughput.
As the flow-rate is the setting used for the pump this research will use this value directly, however, a cell velocity can be calculated with the area of the constriction between the electrodes. Equation 2.1 shows the relation between flow-rate Q, area A and the velocity of a particle. It should be noted that this velocity is higher in the center of the constriction and that the cells will need to accelerate once they enter the constriction, causing them to be slower than this equation would suggest.

$$v_{particle} = \frac{Q}{A} \tag{2.1}$$

Influence of frequency

As stated earlier, it is important to measure the impedance of a passing cell at different frequencies. As the impedance of a passing particle is different at different frequencies, the in-phase and quadrature signals peak heights will change with different frequencies.
Calcified and decalcified cells may respond similarly in one frequency but different in another as the coccoliths will add to the total impedance of the cell. The different makeup in material between the coccoliths and the rest of the cell also creates a difference in the impedance of the total cell. By selecting the right frequencies it should be possible to use the ratio of the different frequency responses to measure the cell type.
Lower frequencies from 1 to around 10 MHz should provide more information on just the size of a cell as the signal cannot pass through the cell membrane, higher frequencies allow the signal to also probe the inside of the cell and provide more information about the makeup of a passing particle.

Chapter 3

Method

The following chapter elaborates on the method of analysis and measurements. A selection of variables will be set at fixed values and the process of measurements and analysis will be shown. Multiple experiments have been conducted in an effort to optimize the final results with different variables.

3.1 Measurement setup

The most important part of the new differtial measurement setup is the impedance measurement chip, this chip is a passive system and on its own it will not provide any useful data. Different systems are added to the measurement setup to generate signals and to control the passing of particles through the channels and past the electrodes. An overview of the different systems, subsystems and transformations is shown in figure 3.1, voltages and currents have been defined from the perspective of the lock-in amplifier.

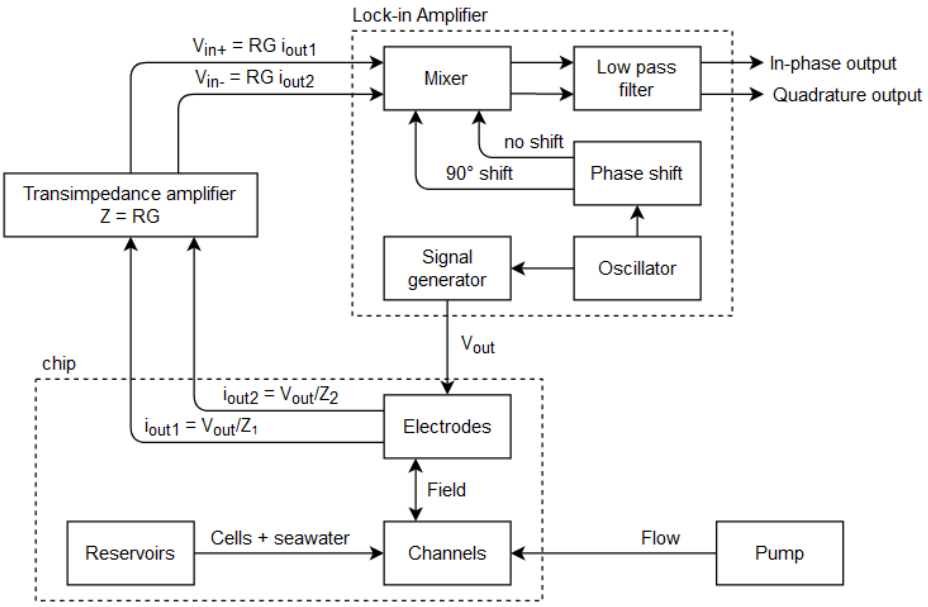

Figure 3.1: *Diagram of the measurement setup*

3.1.1 subsystems

Aside from the glass-PDMS electrode chip the measurement setup has a set of active systems that provide usable signals in the electric domain and flow in the fluid domain. The devices used are listed below. The different parameters these systems introduce are listed in section 3.1.4.

- Lock-in amplifier HF2LI
- Transimpedance amplifier HF2TA
- neMESYS syringe pump
- glass-PDMS chip

The lock-in amplifier and transimpedance amplifier are ran with the LabOne software of Zurich instrument, here different settings can be adjusted and the signal can already be reviewed in real-time.

3.1.2 Electrodes

The three electrodes have been placed around the channel as shown in figure 3.2. The top view of the fluid channels is also shown, at first the channel is $100\mu m$ wide but at the electrodes the channel is constricted to $10\mu m$, this further increases the influence of a passing signal to the total impedance and also limits the amount of particles passing the electrodes at one time. Around the electrodes ground shielding is added to reduce the interaction between the electrodes outside of constriction, resulting in a decrease in parasitic capacitance and interference.

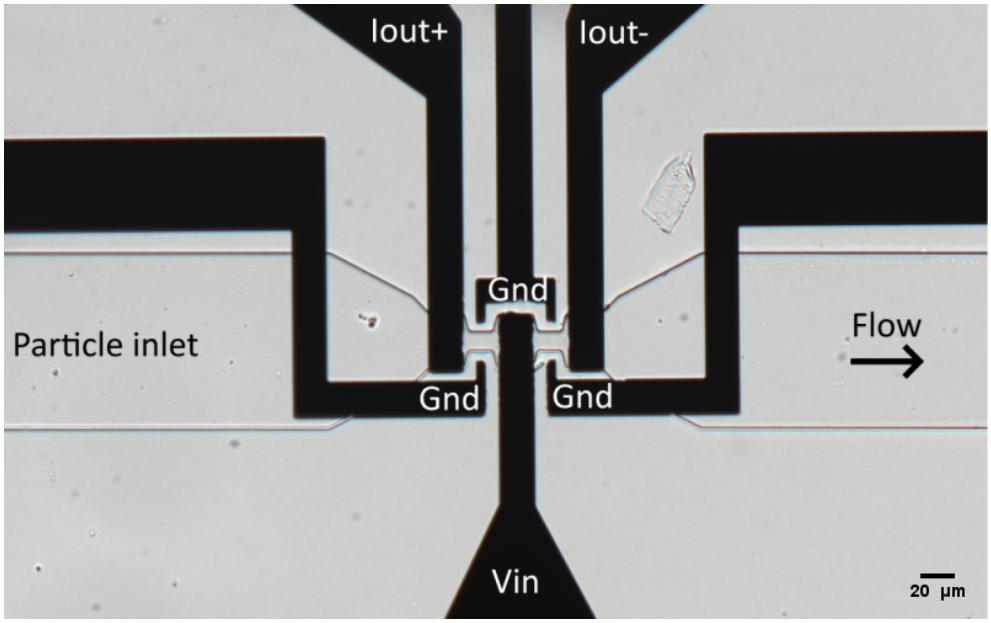

Figure 3.2: *Labeled image of the electrodes and fluid channels*

3.1.3 Polystyrene beads

Different parameters like the pH of the medium can alter the conductivity of the medium, when comparing measurements done on calcified and decalcified cells it is important to either anticipate or counteract the possible changes to the medium that also influence the measured peak heights of cells.
One solution to this is the addition of a third particle that is present in both the calcified and decalcified populations which can then be used to calibrate the measurements.
In this research this third set of particles are polystyrene beads of $5\mu m$ in diameter [1]. These beads are similar in size to the coccolithophores which allows it to be used in the same chip. Beads with $6\mu m$ could also be used if a larger difference in signal response was required but the $5\mu m$ beads showed sufficient differences compared to the coccolithophores.

3.1.4 Variables in the setup

The different subsystems in the measurement setup all have a set of variables than can be adjusted to change the signals caused by a passing particle. Some of these parameters have been selected at the start of this research and others are tested in different experiments. The full list of known parameters is shown below. Other outside parameters can also influence the system like the pH of the medium, temperature, chip alignment and others. Although it is hard to compensate for all of these, most measurements will be conducted using the same chip and in the same session in an attempt to limit these effects.

- Lock-in amplifier
  - Output frequency
  - Output voltage
  - Number of output channels
  - Low pass filter bandwidth
  - Low pass filter order
  - Sampling frequency
- Transimpedance amplifier
  - Amplifier gain R
  - Additional gain G

- Electrodes
  - Spacing
  - Size
- Fluid domain
  - Pump direction
  - Flow
  - Seawater reservoirs addition
  - Cell concentration
  - Polystyrene bead addition

3.1.5 Preset parameters

The following parameters were chosen based on prior knowledge and the provided chip or could be determined using known constraints.

Electrode size

In this research a electrode design is used where the electrodes are spaced $20\mu m$ apart. The width of the electrodes is also $20\mu m$ and the active length of the electrodes at the constriction is $30\mu m$. Further research could inspect the influence of different electrode designs.

Transimpedance amplifier

Two parameters can be set in the transimpedance amplifier settings, the transimpedance gain and an additional gain of 1 or 10. As the operational bandwidth of the HF2TA drops with higher gains the amplifier gain R is limited to 100 V/A or 1000 V/A, using the 1000 V/A the input noise should be smaller as stated in the specifications by the manufacturer [6]. The additional amplification A can be 1 or 10 and both settings could be used, the choice was made to use A of 1 to get a total amplification of 1000 V/A. The transimpedance amplifier has two channels, one for both electrode pairs. These will both be set to the same settings.

Lock-in amplifier

The lock-in amplifier has a larger range of variables, many of these influence the signal in a less predictable amount and are worth testing. There are four settings that can be selected beforehand but may depend on other settings.
The first setting to consider is the low-pass filter bandwidth, a higher order filter will reduce noise past the cut-off frequency more but also influences the settling time. For this research the filter order will be set at 4 but a higher filter order may decrease noise if required in further research.
The second parameter is the sampling frequency, this is also called the data-transfer rate as it is mostly limited by the amount of samples that can be transfered over usb from the lock-in amplifier to the pc on which the lab one software is running. The total available data transfer rate is 460 KSamples per second and has to be divided between the different frequency channels, if two or three channels are used a sampling rate of 115.1 kHz can be used, if more than 4 channels are used the sampling rate will be halved to 57.6 kHz.
The lock-in amplifier allows the use of up to 6 output frequencies at the same time. The use of more frequencies can provide more data on a passing particle, however, two other parameters depend on the channel count. When more channels are used the voltage and sampling rate per channel have to be decreased. Depending on the influence of different frequencies, two or three channels can be selected.
A final parameter that also depends on the number of channels used is the output voltage, the lock-in amplifier can output a total voltage of 10 V which consists of all the individual signals. If 3 frequency channels are used, each channel will be put at 3 V, if 6 frequencies are used, each channel will be put at 1.5 V.

Fluid domain

Different parameters in the fluid domain influence the throughput of cells and the velocity of particles in the channel. The only two settings that can be selected in advance are the cell and bead concentration, concentration of cells in solution slowly changes over time but to maximize throughput the concentration is not lowered. Other parameters in the fluid domain are easy to test and have been tested in a couple of experiments.
The concentration of polystyrene beads will have to be selected to be in a similar range to the concentration of cells in solution, this way a measurement represents both particles sufficiently. On tests that take longer the amount of polystyrene beads can be decreased as a smaller set of beads will then be sufficient to adjust the measurements. As the beads are in high concentration in stock, the bead solution will always be diluted by 5-10 times.

Pump direction

Another choice that can be made in the fluid domain is the direction of the pump flow. It is possible to push or pull fluid through the system. In the three channel system, which will later be introduced fully, pulling is easier as it allows the use of only one pump. A downside to pushing the fluid through the channel is that pushing will increase the pressure inside the chip. An increase in pressure can cause the bonding of the PDMS to the glass-electrode plate to fail earlier. As pulling the fluid is easier and helps prevent the PDMS from releasing from the chip, the pumps are set to pull.

3.1.6 Variables to be selected

The following variables will be tested with a set of experiments, once these variables are all selected it will be possible to do a final test with the optimized variables.

- Output frequency
- Low-pass filter bandwidth
- Flow-rate
- PDMS chip design

3.2 Measurements

The following section will consider each remaining variable and will discuss how the effects of these variables on the signal can be tested. In the next chapter the results of these different measurements will be discussed. As the first tests will be used to characterize the system, only the polystyrene beads will be used. The experiments on frequency response and the test with the final parameters are done to analyse the coccolithophores and will use a mixture of beads and cells.

3.2.1 Bandwidth and flow rate

The first set of experiments tests the effects of the low-pass filter bandwidth and how this changes with different flow rates. This test is done using three frequency channels to test variations over frequency as well. The following settings are selected in the lock-in amplifier:

- Voltage per channel: 3 V
- Sampling rate: 57.6 kHz
- Frequencies: 1, 10 and 25 MHz

The flow-rate used in this test is $0.3\frac{\mu L}{min}$. The area of the constriction is $9\mu m * 10\mu m = 9 * 10^{-12}m^2$ and the flow-rate can be converted to $5 * 10^{-12}\frac{m^3}{s}$. Using equation 2.1 a theoretical velocity of $0.056\frac{m}{s}$ is found. The distance between the centers of the electrode pairs is $40\mu m$ and the time difference between the first and second peak generated by a passing particle will be around 0.7 ms
The best bandwidth for this flow-rate will be measured experimentally and the bandwidth setting for different flow-rates can be elaborated from this. If the flow rate is halved the velocity of a particle will also be about halved, the settling time of the low-pass filter can then be doubled which means the bandwidth can be halved. A lower flow rate would then directly allow the removal of more noise from the signal.
The expected ideal bandwidth will be around 5 to 20 kHz, to get a broader picture of the drop in signal amplitude a wider selection is used. In this experiment the following low-pass filter bandwidths will be tested:

200 Hz	500 Hz	1 kHz	2 kHz	5 kHz	10 kHz	20 kHz	50 kHz

To confirm that the scaling of flow-rate corresponds to the same scaling in bandwidth, a second test is done on bandwidth and flow-rate during the test on additional seawater reservoirs. This was useful partially done to find the change in velocity of particles with the different PDMS layouts as well.

3.2.2 Additional seawater reservoirs

For this differential measurement setup two PDMS chip variants are available. Figure 3.3 shows the two PDMS chip designs. One of these has two reservoirs, one input and one output. The second type has two additional reservoirs next to the input reservoir. These other reservoirs can be filled with seawater and connect to the input channel from the side. This is done to try and constrict the position of particles to the center of the channel, this process is called sheath flow focusing. The position and velocity of the cells should be more consistent using this design. Using the pulling setting of the pump, there is a slight risk that flow

will be different from the three different reservoirs but another feature of the three channel chip helps prevent this, behind each reservoir a constriction is placed that causes a known resistance for fluids to pass, this should help keep the flow equal consistent between the three reservoirs.

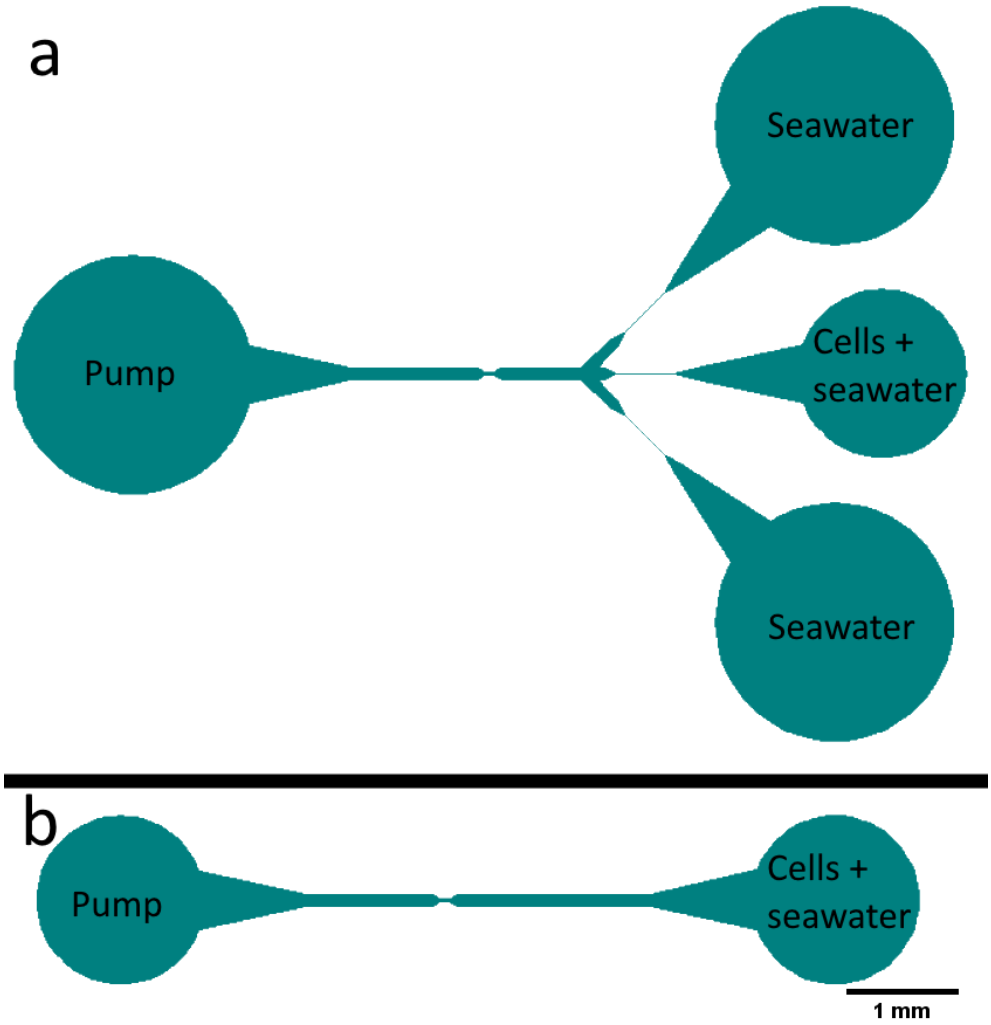

Figure 3.3: *Two types of PDMS chips, the three reservoir design for sheath flow (a) and single reservoir design (b)*

The next set of experiments will be done to compare the two types of PDMS chips. A similar selection of variables is used in comparison to the previous test on bandwidth as the results from those tests can be used as the result for the one reservoir chip. This means only the three reservoir has to be tested again. As the velocity of the three reservoir chip is expected to be faster, the bandwidth is measured again with a smaller selection. The settings used for the comparison between the one channel and two channel chip are:

- Voltage per channel: 3 V
- Sampling rate: 57.6 kHz
- Frequencies: 1, 10 and 25 MHz
- Flow rate: $0.3\mu L/min$
- Bandwidth: 10, 20, 30 and 50 kHz

For the comparison of the same chip with different flow rates the following settings are also used on the three channel chip:

- Voltage per channel: 3 V
- Sampling rate: 57.6 kHz
- Frequencies: 1, 10 and 25 MHz
- Flow rate: $0.1\mu L/min$

- Bandwidth: 5, 10 and 20 kHz

The largest expected difference between the two variants is that the velocity of the particles should become more consistent, as the velocity within a channel is higher in the center of a tube the mean velocity in the three channel chip will be slightly higher and the standard deviation in this time should be lower. To anticipate this change in mean velocity the experiment also uses a smaller set of different bandwidths.
Aside from a decrease in the spread of velocities the results of this experiment will also be used to compare the mean and spread of the signal peak heights.

3.2.3 Output frequencies

The next set of experiments will try to find the frequencies where the difference between the calcified and decalcified cells is largest. The frequencies in the lock-in amplifier can go up to 50 MHz and previous research suggests the use of frequencies of 0.5 MHz and above [2] and frequencies above 30 MHz will mostly by influence by parasitic capacitances. Different frequencies will be tested in the range of 0.5 to 30 MHz. These frequencies will be selected to be spread out over this band in an attempt to sample the impedance spectrum in multiple locations. The full set of frequencies is shown below:

0.5 MHz	1 MHz	3 MHz	4 MHz	6 MHz	10 MHz	20 MHz	25 MHz	27.5 MHz	30 MHz

The lock-in amplifier allows the use of up to 6 different frequencies at one time which means that the 10 frequencies of interest have to be split up in 2 groups. To be able to compare these frequency sets with each other, two frequencies will be present in each of the two sets that can be used as reference. The frequencies have been split in the following groups:

Set 2	1 MHz	25 MHz	4 MHz	6 MHz	20 MHz	30 MHz
Set 1	1 MHz	25 MHz	0.5 MHz	3 MHz	27.5 MHz	10 MHz

Next to these frequencies the following settings have been used, the PDMS variant, flow rate and low-pass filter bandwidth have be selected with the results of the previous tests:
The tests on output frequency were done using the previously determined variables, these parameters are listed below:

- Voltage per channel: 1.5 V
- Sampling rate: 57.6 kHz
- Flow rate: 0.3 $\mu L/min$
- Bandwidth: 30 kHz
- Three reservoir chip
- Frequency set 1: 1, 25, 4, 6, 20 and 30 MHz
- Frequency set 2: 1, 25, 0.5, 3, 27.5 and 10 MHz

These settings were used in two final tests, one with calcified coccolithophores and beads and another with decalcified coccolithophores and beads. The concentration of cells is left as in stock and the beads were added in a ratio of about 1:1. After the measurement was completed the peak heights were evaluated, this was done for both the higher frequencies, 25 MHz and 27.5 MHz, both were compared to the peak heights of the lower 1 MHz signal.

3.2.4 Test with final parameters

A final experiment has been conducted using the now known variables. The test will be conducted over a larger period of time to get a proper amount of samples. This final test can then be used to get an expression of reliability, signal to noise quality and the comparison to previous chips can be made.
In this test the following settings were used based on the results of previous experiments. The choice some of these parameters will be motivated in chapter 4.

- Voltage per channel: 3 V
- Sampling rate: 115.1 kHz
- Flow rate: 0.3 $\mu L/min$
- Bandwidth: 20 kHz
- Single reservoir chip
- Frequencies: 1, 25 and 27.5 MHz

3.3 Analysis

Once different tests have been completed the data has to be processed. Although different tests require some changes in the analysis, a lot of steps are similar for all tests. The following steps have been used to convert the gathered data into peak heights that can be compared with other experiments. This analysis is conducted in MATLAB where a range of functions is available to help in different parts of the analysis. In the appendix multiple important chunks of the code used in the analysis can be found, the following sections will explain how this code was designed and used.

3.3.1 Baseline removal

The first step in processing the data is to center the signals around zero. This should not be necessary according to the expected signal but asymmetry between the electrodes and a difference in pressure at the two points of measurement cause a slight offset in the differential signal. This offset is seen to slightly change over time, to best remove this offset a second order polynomial fit is made and subtracted from the original signal.

3.3.2 Peak detection

Once the signal has been centered around zero it is necessary to detect peaks present in this signal. A standard function is present in MATLAB to find the peaks in a signal. Two requirements to this function have been added to improve the accuracy of this function.
The first parameter is the minimum distance between the peaks, this minimum distance can be adjusted to set a certain area around each peak where no other peaks are allowed to exist. This parameter depends on the sampling frequency as this parameter sets a boundary in samples, not in time.
The second parameter is the minimum peak height, this parameter is set to prevent noise from being detected as a peak, it is important to adjust this parameter to the noise levels present in the signal which can change with voltage and low-pass filter bandwidth.
Using this function on the in-phase and quadrature signal of each frequency separately will find most peaks but when a peak is not found in one signal but where it is found in another it makes it difficult to link the different peaks to each other. To counteract this the findpeaks() function is used only on the signal with the largest peak height. In the initial test this is the in-phase signal at 25 MHz. The other peaks are found by selecting the local minima and maxima in the remaining signals. This way it is also possible to link the positive and negative peaks generated by a single particle.
Figure 3.4 shows an example of a signal generated by passing particles, this example is taken from the measurements on the influence of different frequencies. The figure shows the different responses at different frequencies, some peaks first show a positive peak and a negative peak after and some show a negative peak first and a positive peak after, this shows that at some frequencies the particle has a higher impedance than the seawater and at some frequencies it has a lower impedance.

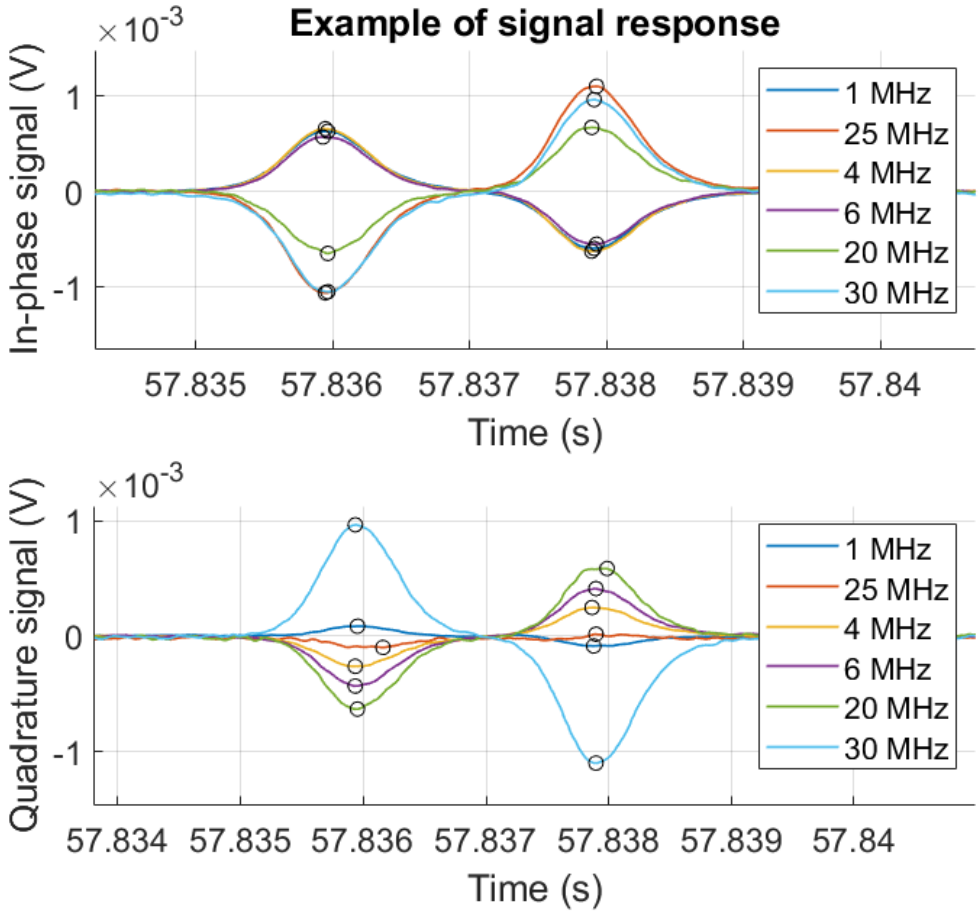

Figure 3.4: *Example of measured peaks and detected peak heights*

3.3.3 Normalization of peaks

In later tests the peak heights at different frequencies turned out to be difficult to compare directly. As the impedance response of beads is known to be constant in tests done with calcified and decalcified cells, an algorithm is implemented that normalizes the peak heights present in an experiment. The normalization is done for each frequency separately, for both the in-phase and quadrature signals.
As peaks generated by multiple particles can distort the normalization by appearing as really large beads, any particle that has an opacity or peak height that deviates more than 25% from the first mean of the beads is removed and the beads are normalized once more. This is possible as previous tests showed that the peak heights of beads deviate about 10% of the mean at most.

Separation of beads and cells

When both beads and coccolithophores are present in an experiment it is important to seperate these two groups before both groups can be normalized using the beads. To do this the k-means function in MATLAB is used. This function takes the responses of particles at different frequencies and tries to split these into k groups. In these experiments two groups are present, beads and cells.
Figure 3.5 shows an example of the peak heights generated by different particle types which are separated into two groups.

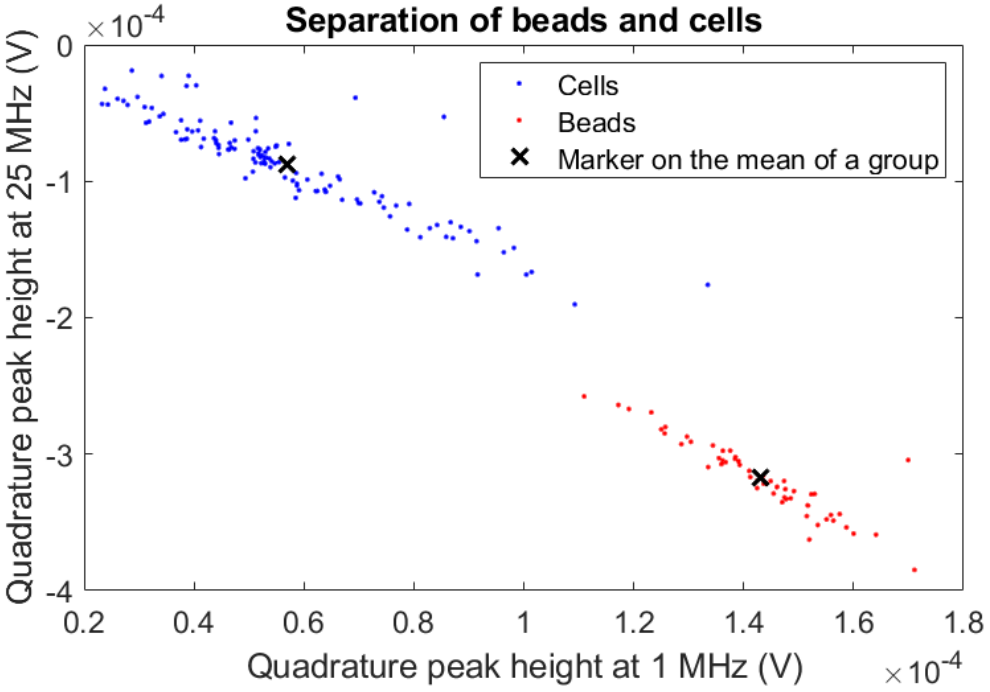

Figure 3.5: *K-means separation of cells and beads using the 1 and 25 MHz peak heights*

3.3.4 Opacity

Once the peaks of the different signals have been found and normalized the peaks in different tests can be compared. As stated before, the use of only one frequency does not provide enough information to separate cells into calcified and decalcified because of the influence of cells size. A way to counteract the influence of cell size is the introduction of opacity. Opacity is the ratio of the in-phase or quadrature peak height at two different frequencies. The cells are measured both at a frequency where the response is heavily dependent on cell size and at a frequency where cell size is less important. The opacity can then be used to separate cells in their calcification state.

Chapter 4

Results and discussion

4.1 Bandwidth and flow rate

One test was done to test the best bandwidth setting using a set flow-rate. The best bandwidth is the bandwidth where the full peak of a passing particle is measured with the lowest possible noise. The noise is quantified by measuring the standard deviation in peak height.

4.1.1 Bandwidth

Once the peak height of passing beads was measured using different low pass filters in different measurements. The average peak height and standard deviation of the peaks is shown in figure 4.1, here it can be seen that using a bandwidth of 5 kHz and higher all create a similar average peak height. The 20 kHz setting had the best standard deviation compared to the mean peak height and is chosen for further tests with this flow-rate.

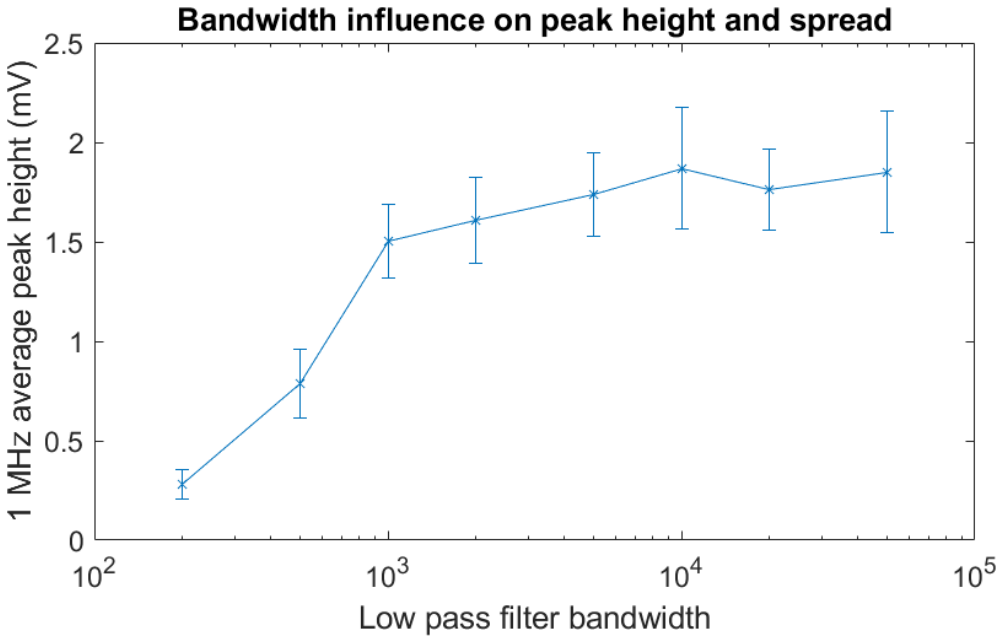

Figure 4.1: *Influence of low pass filter bandwidth on peak height*

4.1.2 Flow rate

The actual velocity of passing particles is measured by looking at the time between the positive and negative peaks of a passing particle. The average time between peaks in the 20 kHz bandwidth experiment was found to be $0.905ms$. This corresponds to a velocity of $0.044\frac{m}{s}$, as expected this velocity is slower than the theoretical value of $0.056\frac{m}{s}$ as the theoretical velocity is the average velocity of the seawater and cells passing through the constriction.
This measurement was done using the $0.3\frac{\mu L}{min}$ flow-rate setting, the resulting signal had a decent throughput with an average of 15 beads per second passing the electrodes. This throughput does depend on the concentration of the beads and later also the concentration of cells in the reservoir. When a lower flow rate is selected the amount of particles measured per second goes down but the time of each passing particle goes up. A lower flow-rate would generate more samples on a single peak and will allow a lower bandwidth and with that a better signal to noise ratio.

4.2 Fluid channel count

Before the effects of the sheath flow focusing in this three reservoir chip can be analysed, it is necessary to recalibrate the bandwidth for use with the new chip, after the best bandwidth was found for use with this chip, the results could be compared fairly with the one reservoir chip.

4.2.1 Bandwidth and flow-rate

During the experiment on the chip with three fluid reservoirs, measurements were done using a flow-rate of $0.1\frac{\mu L}{min}$ and $0.3\frac{\mu L}{min}$. First, different bandwidths were tested and compared, for the $0.1\frac{\mu L}{min}$ test, a bandwidth of 10 kHz had the smallest standard deviation in the peak height and for the $0.1\frac{\mu L}{min}$ test the best bandwidth turned out to be 30 kHz.
The average time between the positive and negative peaks of a particle was found to be 0.76 ms with a flow-rate of $0.3\frac{\mu L}{min}$, which corresponds to an average velocity of $0.0526\frac{m}{s}$, which is closer to the theoretical $0.0567\frac{m}{s}$ than the $0.044\frac{m}{s}$ of the chip without flow focusing. The new chip reached 92.8% of the theoretical velocity where the old chip had an average of 77.6%.
Next to the change in chip, the effects of changing the flow-rate on the best bandwidth was also tested. The average time between peaks using a flow-rate of $0.1\frac{\mu L}{min}$ is 2.20 ms and the corresponding velocity is $0.0182\frac{m}{s}$.The cell velocity in the $0.3\frac{\mu L}{min}$ measurement was 2.89 times that of the $0.1\frac{\mu L}{min}$ cells. The theoretical velocity using a flow-rate of $0.1\frac{\mu L}{min}$ is $0.0185\frac{m}{s}$ and the measured velocity is now 98.4% of the theoretical velocity. That this measured velocity is closer to the theoretical velocity is possible since the cells go slower and cause less turbulence.
The peak spacing of the two three reservoir tests and the previous one reservoir test can be compared with figure 4.2. The first comparison can be made between the single reservoir measurements and the three reservoir measurements. Since the beads were concentrated to the center of the channel, a higher average velocity was expected and this can also be seen in figure 4.2. The flow focusing should also make the velocity more predictable this way and this can also be seen in a decrease in standard deviation from the mean, this shows that the sheath flow focusing works as intended and causes the velocity of passing particles to be more predictable.
The second comparison that can be done is between the two flow-rate settings on the three reservoir chip, what can be seen is that the average peak spacing is almost three times longer when the flow-rate is decreased. The $0.1\frac{\mu L}{min}$ measurements appear to be less consistent but the standard deviation in peak spacing σ can be divided by the corresponding mean μ to get a better comparison. For the $0.1\frac{\mu L}{min}$ measurement the coefficient of variation $CV = \frac{\sigma}{\mu} * 100\%$ is 6.24% and for the $0.3\frac{\mu L}{min}$ measurement it was 5.66%. This does mean that the velocity is a bit more stable at a higher flow-rate. The flow-rate for following tests is set at $0.3\frac{\mu L}{min}$ as this flow-rate is a good trade-off of throughput and signal quality.

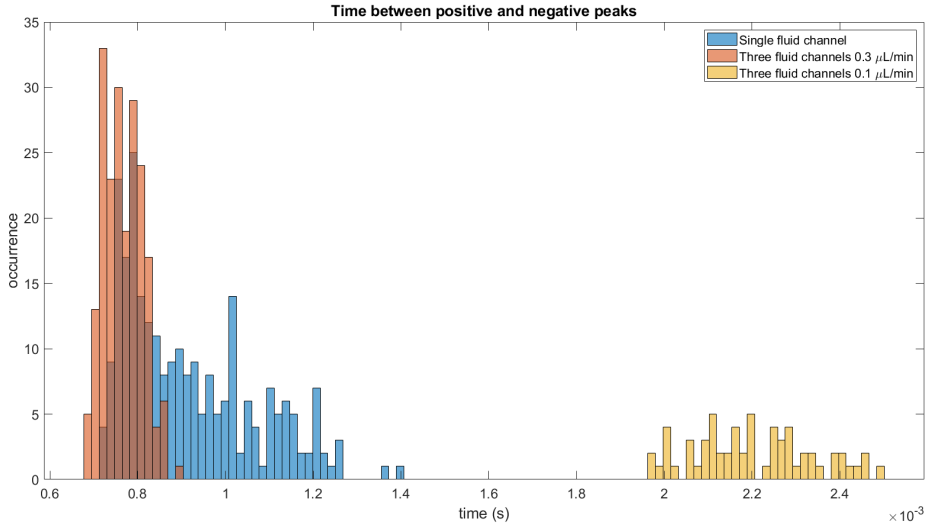

Figure 4.2: *Comparison of the time between the positive and negative peaks of a passing cell*

The peak heights of the one and three reservoirs chips is also compared to see if the addition of sheath flow focusing improves the consistency of measurements of the peak height. A histogram of the peak heights is shown in figure 4.3, as can be seen here the spread in the in-phase 1 MHz peak heights did not improve much, the coefficient of variation in the one reservoir chip is 11.5% and that of the three reservoir chip is 9.65%. The coefficient of variation of the peak heights of the in-phase and quadrature signals of the 10 and 25 MHz measurements that were also done all showed a similar difference of about 2%. It can be concluded that the use of sheath flow focusing does improve the quality of the measurements.

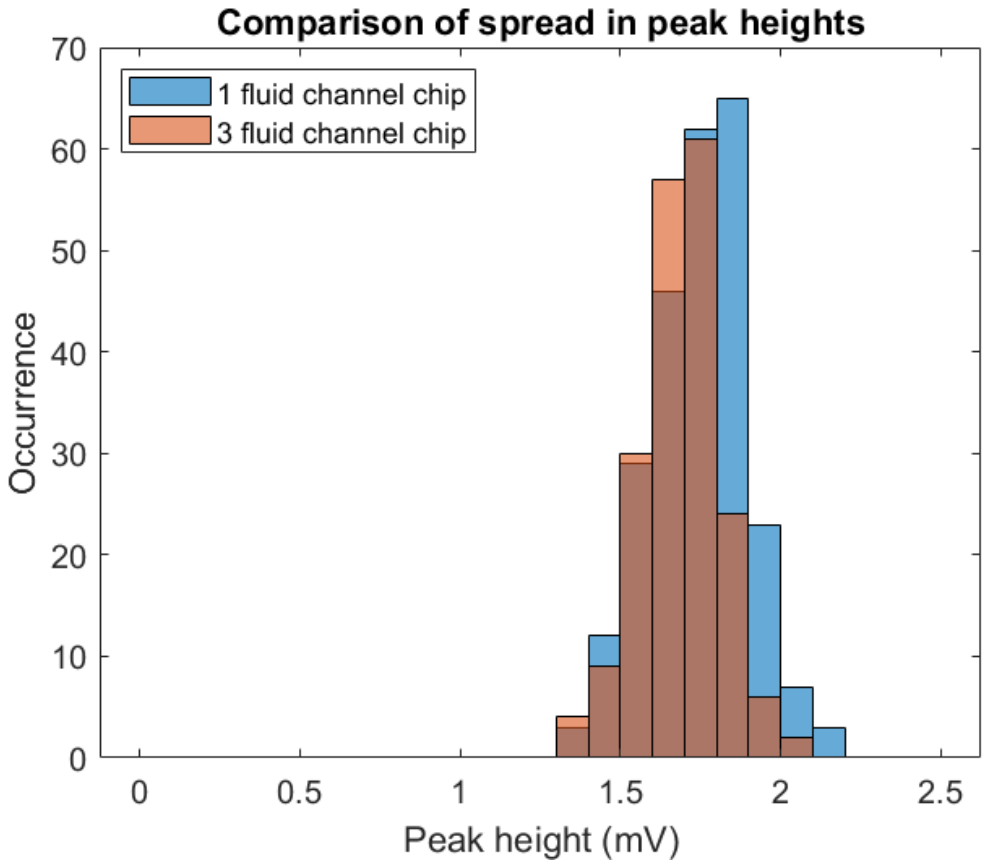

Figure 4.3: *Comparison the 1 MHz peak height of the one and three reservoir chip designs*

4.3 Output frequencies

Once the two frequency sets were measured, the opacity in comparison to 1 MHz was made for the in-phase and quadrature signals. This collection of opacities was averaged and then used to compare calcified and decalcified signals. The following relative differences in opacity were found:

Frequency (MHz)	1	0.5	3	4	6	10	20	25	27.5	30
Change in quadrature opacity %	0	-0.2	-4.4	-0.34	0.25	0.18	2.4	13.4	-18.4	-1.9
Change in in-phase opacity %	0	-0.15	0.1	-0.02	0.4	3.1	-1.3	0.4	2.5	2.2

The two largest differences in opacity appear at the quadrature opacities at 25 and 27.5 MHz, however, some smaller differences also exist in both the in-phase and quadrature signals. .
Aside from the average opacity to get a measure of spread in the opacity the standard deviation in the opacity was calculated. The in-phase opacity means and standard deviations can be seen in figure 4.4 and the quadrature variant can be seen in figure 4.5. The 25 MHz opacity is measured twice as it was present in both frequency sets. The 1 MHz opacity is one by definition as the opacity was created with the 1 MHz signal.
Using the quadrature opacities at 25 and 27.5 MHz the coccolithophores can be also separated from the beads easily as the opacities are either much larger or smaller than 1. The in-phase opacities of the cells are all closer to that of the beads. Because of the large separation in opacity between the beads, calcified cells and decalcified cells, the selected frequencies are 1, 25 and 27.5 MHz. The 1 MHz signal can still be replaced with another smaller frequency but as it was already present in all other tests it allows the comparison of the final test with all previous tests.

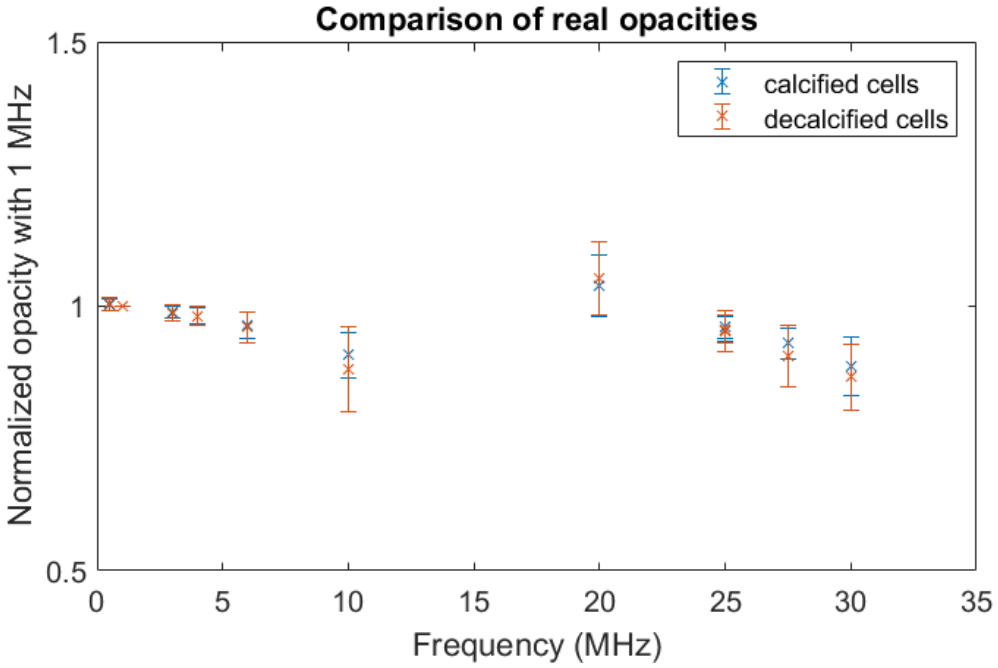

Figure 4.4: *Comparison of in-phase opacities with the different measured frequencies in the numerator and 1 MHz as denominator, the mean is showed as an x and the bars show the standard deviation in opacity.*

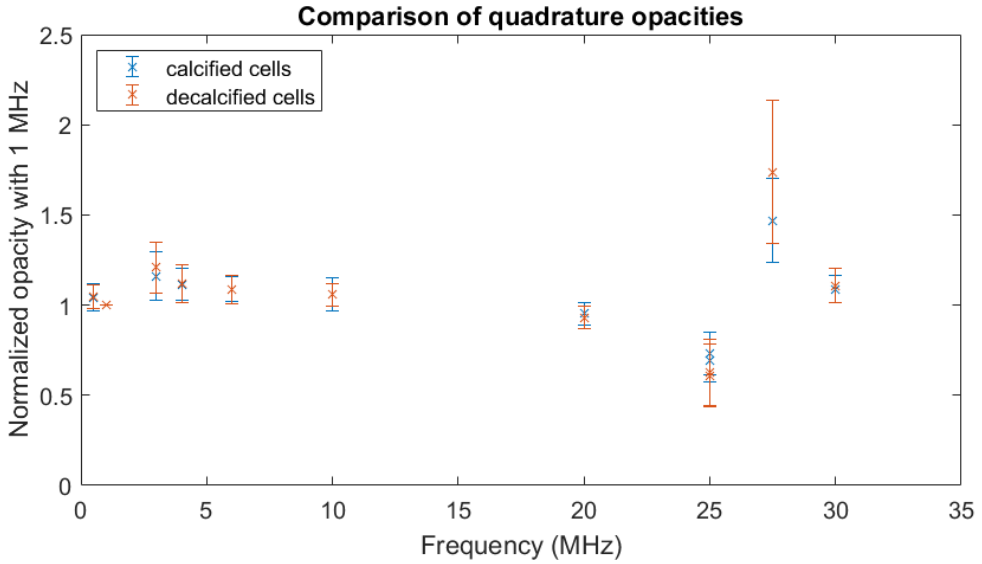

Figure 4.5: *Comparison of quadrature opacities with the different measured frequencies in the numerator and 1 MHz as denominator, the mean is showed as an x and the bars show the standard deviation in opacity*

4.4 Final test

Once all the previous experiments were processed a set of final variables was selected. Before normalization the opacity of both beads and cells showed interesting values in the 25 MHz signal, the quadrature peak heights at 25 MHz were about 50% smaller than in previous measurements. Although the normalization of cells in comparison to the beads was introduced to compensate for this, previously the variation in peak heights were much smaller. This suggests a problem with the 25 MHz signal. Luckily, the 27.5 MHz and 1 MHz signals were similar to previous measurements. Figure 4.6 shows the quadrature peak heights at 25 MHz compared to the 1 MHz peak height. Figure 4.7 shows the same plot but using the 27.5 MHz and 1 MHz signals.
The 25 MHz and 1 MHz figure is clearly more chaotic than the 27.5 and 1 MHz figure, this is again caused by the small peak height at 25 MHz and the resulting poor signal to noise ratio.
An important thing to notice is a group of cells in the decalcified measurement that overlap with the opacity of the calcified cells. This overlap means that the decalcified solution was contaminated with calcified

cells at some point. As the decalcified test was done before the calcified test in the chip it is likely that not all cells were decalcified correctly.

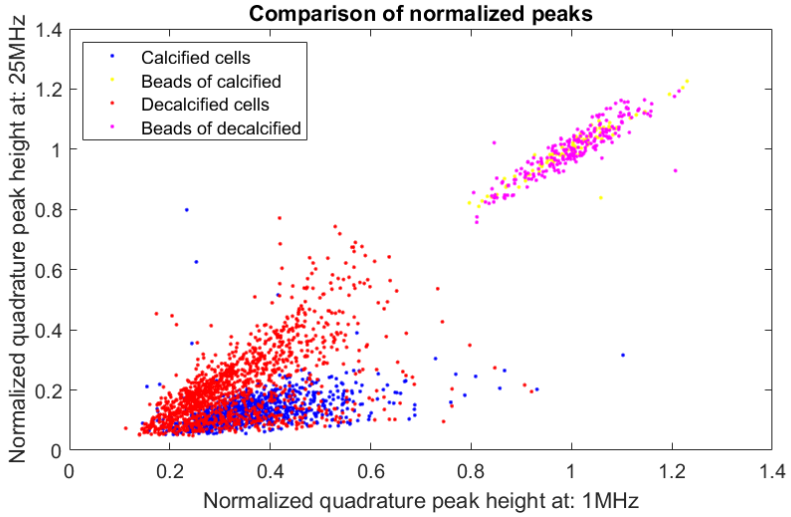

Figure 4.6: *Normalized quadrature peak height of 25 MHz and 1 MHz signal for calcified and decalcified cells mixed with beads*

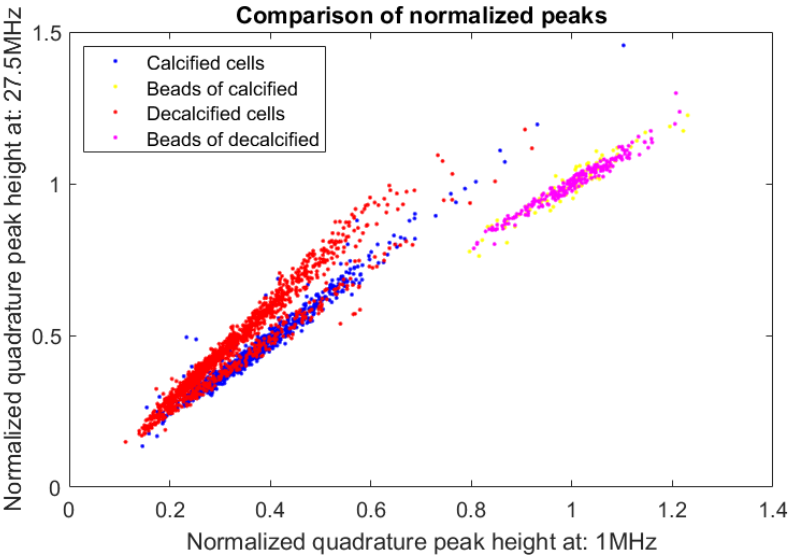

Figure 4.7: *Normalized quadrature peak height of 25 MHz and 1 MHz signal for calcified and decalcified cells mixed with beads*

After finding and normalizing the peaks, the opacity was calculated for both 25 and 27.5 MHz with 1 MHz as reference for cell size. The opacity of both the in-phase and quadrature signals have been calculated and can be evaluated separately. The relative difference in quadrature mean opacity between the calcified and decalcified measurements was calculated to be an increase of 14.8% at 27.5 MHz and an increase of 96.7% at 25 MHz. As noted before, the 25 MHz signal did not behave as expected, instead of a 11% decrease in opacity of the decalcified cells compared to the calcified samples, there was a much larger increase in mean opacity, the standard deviation of the 25 MHz quadrature opacity is much larger than that of the 27.5 MHz signal as well. This further supports the theory that the 25 MHz signal was obstructed in some form.
Two histograms are also made to compare the opacities of calcified and decalcified cells. The opacities of the beads are shown to be centered around 1, which is a measure of the success of normalizing the signals. The 25 and 1 MHz opacity histogram can be seen in figure 4.8, the quadrature opacity of the decalcified cells was expected to be smaller than that of the calcified cells but instead shows to be larger, the opacity is also very spread out, this wide spread in the opacity is partially caused by the smaller peak height at 25 MHz which decreases the signal to noise ratio.
The 27.5 and 1 MHz opacity histogram already shows a decent separation of the two calcification states. There is however, still an overlap between the two groups. The decalcified measurements appear to consist of two groups here as well, one group overlaps the histogram of the calcified cells and the other group is to

the right of both the first decalcified group and the calcified cells. This again shows that the decalcified cells could have been contaminated with calcified cells.

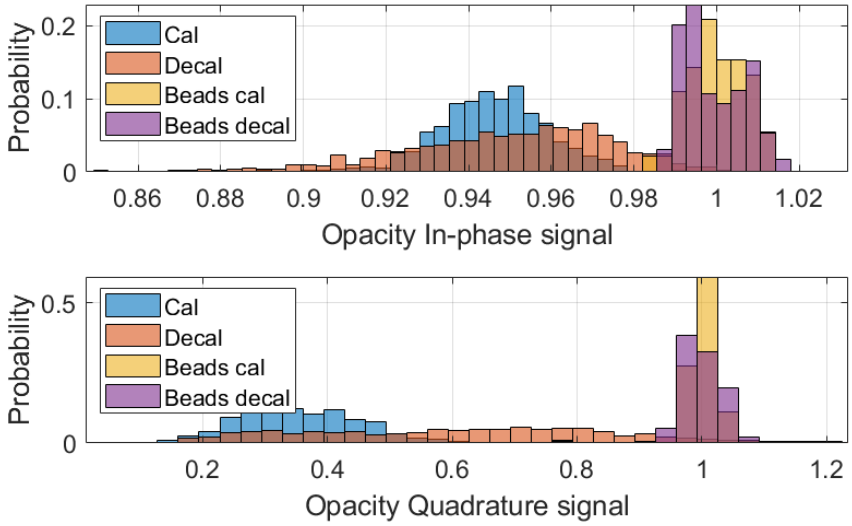

Figure 4.8: $\frac{25}{1}$ *MHz Opacity measurements of both calcified and decalcified cells with beads*

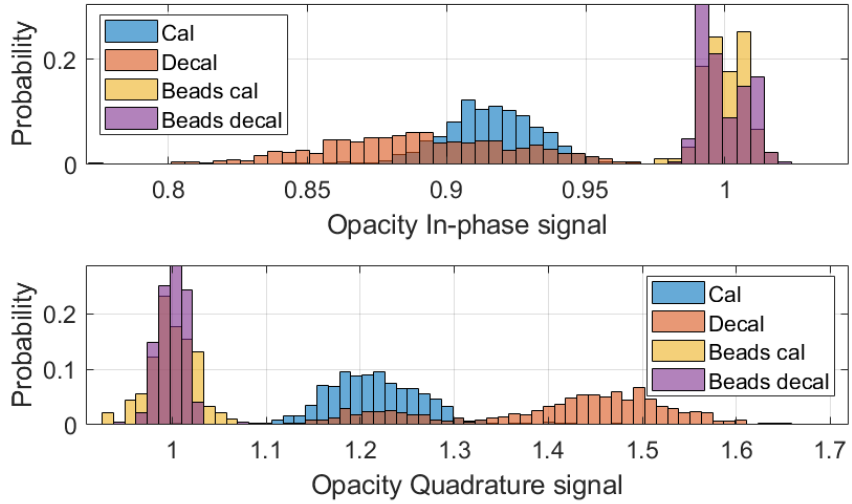

Figure 4.9: $\frac{27.5}{1}$ *MHz Opacity measurements of both calcified and decalcified cells with beads*

By implementing another k-means separation using the quadrature 27.5 and 1 MHz opacity, the assumed contamination of calcified cells can easily be separated from the other, decalcified cells in the measurement. Figure 4.10 shows the two groups found in the decalcified cells measurement. When peak heights are smaller the groups start to overlap slightly and the separation might not be fully correct, however, at larger peak heights the two groups separate completely and are visually distinct.
The 27.5 and 1 MHz mean quadrature opacity of the contamination in the decalcified cells is 1.231 and the standard deviation in that opacity is 0.061. The mean of the same opacity of the calcified measurements is 1.224 with a standard deviation of 0.08. Finally the now separated decalcified cells have a mean opacity of 1.472 with a standard deviation of 0.062.
The opacity of the calcified measurements and the contaminated group in the decalcified measurement are very similar and supports the theory the decalcified sample also contained a set of calcified cells

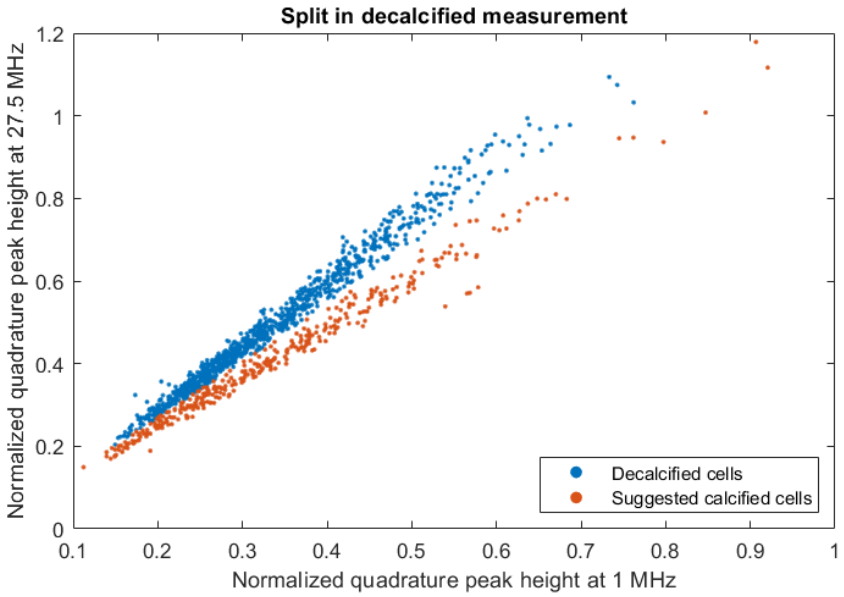

Figure 4.10: *27.5 MHz and 1 MHz peak height comparison with k-means separation of two groups*

It is now also worth looking at the measurement without the calcified group in the decalcified measurement. The 27.5 and 1 MHz quadrature opacity histogram can be seen in figure 4.11, the quadrature opacity at this frequency shows a clear separation of the calcified and corrected decalcified cells, the difference in average opacity is now as expected from the tests on frequency suggested which showed a 18.4% increase in opacity, compared to the 20% increase in opacity in this experiment after correction of the decalcified measurement.

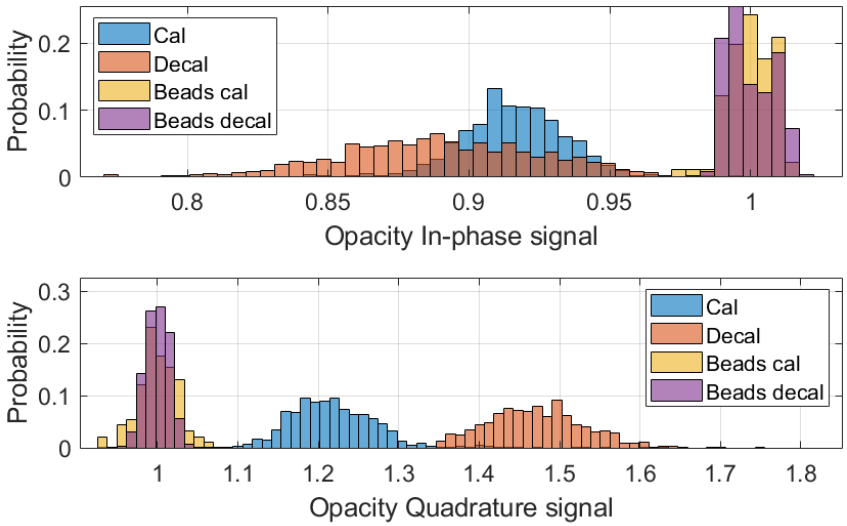

Figure 4.11: $\frac{27.5}{1}$ *MHz Opacity measurements of both calcified and decalcified cells with beads after removal of the second group of cells*

4.4.1 Quality of measurements

To get a measure for the quality of the measurements it is useful to look at the noise levels and how these compare to the peak heights. In the final test Figure 4.12 shows an example of the signal of a passing peak. This particular peak was one of the smallest peaks allowed by the peak height threshold in an effort to show the worst possible influence of noise. The baseline of the 27.5 and 25 MHz quadrature signals is not completely zero which can happen when the baseline changes during the measurement and the baseline removal does not remove this completely as it takes a measure of the average baseline over the entire measurement. In the real signal the influence of noise is small, the height of the noise when no particle is passing the electrodes is about $15\mu V$ for the real 25 MHz signal, a peak generated by the same signal is $450\mu V$ or larger, the noise is then 3% of the peak height. For the quadrature signals the noise is always a bit worse as most quadrature peaks are smaller. For the 25 MHz quadrature peak in the example the noise completely distorts the peak signal, this again is weird as in previous measurements the 25 MHz quadrature peaks were larger.

For the 1 MHz quadrature peaks that were used to get the final result the peak height is $92\mu V$ or higher and the height of the noise is $7\mu V$, this is 7.6% of the peak height.

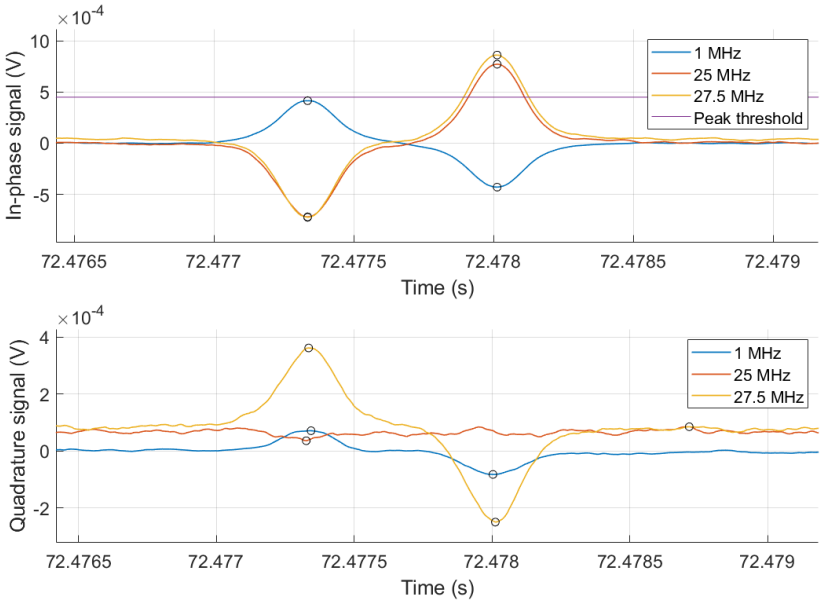

Figure 4.12: *Example of the signal response of a measured cell*

Another requirement for the reliability of the tests is the amount of particles that have been measured. In the decalcified measurement, 890 beads and 1279 cells were found and in the calcified measurement 91 beads and 852 cells were found, the ratio of beads and cells should have been similar but in the calcified measurement many beads got stuck at the inlet of the channels which caused a decrease in beads through the constriction. The amount of cells in both measurements was at least sufficient and through the number of cells the influence of noise in the peak height of individual cells is reduced.

4.4.2 Separation in calcification state

Once the signals of the final test were analysed a separation in calcification state appeared to exist, however, the two groups in the decalcified measurements still pose a problem. Since it is highly likely that this second group was contamination of calcified cells it was also worth looking at the results after the contamination was removed from the measurements.
After the removal of the second group in the decalcified measurements the results of the final measurement using the 27.5 and 1 MHz signals show a separation of the calcified and decalcified cells at a normalized quadrature opacity of 1.34, this suggests that this analysis could be used with an unknown mixture of calcified and decalcified cells and an accurate estimate of the calcification state can be made.

4.4.3 Limitations in the setup

The largest limitation in this measurement setup is the inconsistency between different tests. The contamination in the decalcified cells and the unexpected behaviour at 25 MHz are examples of this. At the moment the placement of chips and the quality of bonding between the glass and the PDMS also changes from test to test, this makes it harder to do consistent measurements. At the moment the polystyrene beads are also still required to calibrate measurements, ideally, tests would be consistent enough that the beads are no longer needed, however, the differential measurements have shown to be significantly influenced by external factors and small variations will always exist.

Chapter 5

Conclusion

This report has shown the process of testing and selecting different variables in a system that worked toward a final experiment on classifying the calcification state of calcifying algae using differential impedance measurements.
The tasks that were set in the project plan have all been completed but not all of them have had ideal results. The final test was not as clean as it could have been but the result was still useful.
The lock-in amplifier low-pass filter bandwidth, fluid flow-rate and lock-in amplifier output frequency settings were researched and selected with different requirements.

5.1 Research question

Does the introduction of differential measurements allow for reliable classifications of the calcification state of calcifying algae?

The research question can be separated in two parts, is this method for classifying the calcification state reliable and what is the quality of this classification.
The final test still came with some problems, these were contamination and the unexpected values for the 25 MHz measurements. This means that the method was not 100% reliable as is. If more time was available, the same test could have been done again and these problems might not be present in a new test. Another, more consistent problem with the differential measurement is that normalization has to be done on the measurements using the beads, although this has been effective in compensating for changing responses between measurements it makes it hard to set distinct values on opacities that correspond to the calcification state of a cell.
The second question is regarding the quality of the measurements. With the final test it was possible to separate the decalcified and calcified cells into two groups of opacity, this means that the signal quality was high enough for the purpose of classifying cells into these two groups. This classification does become more reliable when larger groups of cells are measured as it cancels out possible variations in individual cell responses due to noise.

5.2 Further recommendations

The first step in improving this research is an additional measurement using the final test settings in an attempt to overcome the problems that occurred in the experiments done during this research.
This next step also builds on the first, the repeatability of tests remains a problem and should be worked on. Classifying the calcification state using a differential measurement system appears to be a working solution but information on the total impedance between the electrodes is lost and could be helpful in comparing the consistency between results.
A couple of influences on this system have not been tested yet and can still be. The first is the influence of time after decalcification of the cells, if the medium with decalcified cells is recently decalcified the cells have not had time to regrow coccoliths yet and are fully decalcified, however the pH of the solution may not have fully recovered yet at this point, this can influence the measurements in a yet unknown way.
A second change that may be worth exploring is the inversion of input and output electrodes. When the two outer electrodes are used both electrodes can now use a different 10 V output range of the lock-in amplifier which could improve the results.
A final improvement to the research could be refining the values used for different parameters. Some settings like the bandwidth and frequencies were tested in a rough selection, for a flow-rate of $0.1\frac{\mu L}{min}$ in the three input reservoir chip design a bandwidth of 10 kHz was shown to be better than 5 and 20 kHz but a bandwidth of 15 kHz might be even better. The same goes for the frequencies used for the measurements. Currently the 1 MHz frequency is used and could be replaced with other frequencies between 0.5 and 10 MHz without changing the results much, but the 25 and 27.5 MHz frequencies could also be refined to improve results.

Bibliography

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Chapter 6

Appendix

6.1 Code on baseline removal

```
for ch = 1:nchannels
    % Fit a polynomial of order 'order' and remove this from the data
    [p,~,mu] = polyfit((1:numel(d(ch).real))',d(ch).real,order);
    f_y = polyval(p,(1:numel(d(ch).real))',[],mu);
    d(ch).real = d(ch).real - f_y;        % Detrend real data
    clear f_y

    [p,~,mu] = polyfit((1:numel(d(ch).imag))',d(ch).imag,order);
    f_y = polyval(p,(1:numel(d(ch).imag))',[],mu);
    d(ch).imag = d(ch).imag - f_y;        % Detrend imaginary data
    clear f_y
end
```

6.2 Code on peak detection

```
%% Peak detection

% Use the findpeaks function to find the peaks in pchR, often 25 MHz
% in-phase signal, multiplied with signR to find the first peak in time
[d(pchR).dRh1,d(pchR).dRlocs1] = findpeaks(signR(pchR)*d(pchR).real(1:end-min_distance),'MinPeakHeight',min_re
    'MinPeakDistance',min_distance);

% Remove first peak in case its too close to the beginning of the signal
d(pchR).dRlocs1(1) = [];
d(pchR).dRh1(1) = [];

% Reintroduce the sign to the peak
d(pchR).dRh1 = d(pchR).dRh1*signR(pchR);

% Find the additional peaks
pcount = numel(d(pchR).dRlocs1); % Number of peaks

for ch = 1:nchannels

    if (ch== pchR) % Skip the peaks already found for this channel
        [d(ch).dRlocs2, d(ch).dRh2] = findAdditionalPeaks(d(pchR).dRlocs1,d(ch).real,-signR(ch), pcount, min_d
        [d(ch).dIlocs1, d(ch).dIh1] = findAdditionalPeaks(d(pchR).dRlocs1,d(ch).imag,signI(ch), pcount, min_di
        [d(ch).dIlocs2, d(ch).dIh2] = findAdditionalPeaks(d(pchR).dRlocs1,d(ch).imag,-signI(ch), pcount, min_d
    else
        [d(ch).dRlocs1, d(ch).dRh1] = findAdditionalPeaks(d(pchR).dRlocs1,d(ch).real,signR(ch), pcount, min_di
        [d(ch).dRlocs2, d(ch).dRh2] = findAdditionalPeaks(d(pchR).dRlocs1,d(ch).real,-signR(ch), pcount, min_d
        [d(ch).dIlocs1, d(ch).dIh1] = findAdditionalPeaks(d(pchR).dRlocs1,d(ch).imag,signI(ch), pcount, min_di
        [d(ch).dIlocs2, d(ch).dIh2] = findAdditionalPeaks(d(pchR).dRlocs1,d(ch).imag,-signI(ch), pcount, min_d

    end
end

% Average peak heights and peak locations
for ch = 1:nchannels
    d(ch).dRlocs = round((d(ch).dRlocs1+d(ch).dRlocs2)/2);
```

```
    d(ch).dRh    = (d(ch).dRh1-d(ch).dRh2)/2;
    d(ch).dIlocs = round((d(ch).dIlocs1+d(ch).dIlocs2)/2);
    d(ch).dIh    = (d(ch).dIh1-d(ch).dIh2)/2;
end
```

6.2.1 Find additional peaks function

```
function [plocs, phs] = findAdditionalPeaks(x,y,sign,pcount,wsize)
    %% Finding local maxima as additional peaks
    % Rutger Kersjes
    % pcount -> number of peaks
    % x -> peak locations
    % y -> signal where peaks need to be found
    % sign -> function finds maxima, for negative peaks the sign is -1
    % wsize -> window size in which peaks can be found

    % initialise output
    plocs = zeros(pcount,1);
    phs = zeros(pcount,1);

    % For every peak, find local maximum
    for p = 1:pcount
        ploco = x(p); % Location of peak
        [~,ind] = max(sign*y(ploco-floor(wsize/2):ploco+floor(wsize/2))); %Find location of local maximum
        plocs(p) = ind+ploco-floor(wsize/2)-1; % Store location of new peak
        phs(p) = y(plocs(p));   % Store height of found peak
    end
end
```

6.3 Code on normalizing peaks

```
%% Peak normalization
% Rutger Kersjes

clc
clear variables
close all

% Load a set of peak data
load('m14_cal_f1.mat');

% Find the number of frequency channels in the data
nchannels = size(R_peaks,2);

%% Split into groups
[idx,C] = kmeans(I_peaks(:,1:2),2); % Find groups based on the first two quadrature signals
if (C(1,1) > C(2,1))    % Beads group has a larger mean peak height
    b = 1;
    c = 2;
else
    b = 2;
    c = 1;
end

% Split the data set into the cells and beads
cellsR = R_peaks(find(idx == c),:);
beadsR = R_peaks(find(idx == b),:);
cellsI = I_peaks(find(idx == c),:);
beadsI = I_peaks(find(idx == b),:);

%% First normalization
Rmean = mean(beadsR); % In-phase mean bead peak height
Imean = mean(beadsI); % Quadrature mean bead peak height

% Initialize variables
cellsRN = zeros(size(cellsR));
beadsRN = zeros(size(beadsR));
```

```
cellsIN = zeros(size(cellsI));
beadsIN = zeros(size(beadsI));

% Normalize each frequency channel
for ch = 1:nchannels
    cellsRN(:,ch) = cellsR(:,ch)/Rmean(ch); % Normalize in-phase peaks of cells
    beadsRN(:,ch) = beadsR(:,ch)/Rmean(ch);
    cellsIN(:,ch) = cellsI(:,ch)/Imean(ch);
    beadsIN(:,ch) = beadsI(:,ch)/Imean(ch); % Normalize quadrature peaks of cells
end

%% Remove faulty bead peaks and renormalize
% Find and remove beads that deviate from the mean further than an actual
% bead would
ind = find((beadsRN(:,1) < 0.75) | beadsRN(:,1) > 1.3 | abs(beadsIN(:,2)./beadsIN(:,1) - 1) > 0.1);
beadsRN(ind,:) = [];
beadsIN(ind,:) = [];

Rmean = mean(beadsRN);  % Recalculate the normalization ratios
Imean = mean(beadsIN);

for ch = 1:nchannels
    %Renormalize the different peaks with the corrected bead mean peak
    %heights
    cellsRN(:,ch) = cellsRN(:,ch)/Rmean(ch);
    beadsRN(:,ch) = beadsRN(:,ch)/Rmean(ch);
    cellsIN(:,ch) = cellsIN(:,ch)/Imean(ch);
    beadsIN(:,ch) = beadsIN(:,ch)/Imean(ch);
end
```

6.4 Code on comparison of normalized peaks

```
%% Comparison of normalized peaks
% Rutger Kersjes & Douwe de Bruijn
% 2-12-2020
%v1

clc
clear variables
close all


% Select the set of frequencies used in the experiment
%freq = ["1MHz", "25MHz", "4MHz","6MHz","20MHz","30MHz"];
%freq = ["1MHz", "25MHz", "0.5MHz","3MHz","27.5MHz","10MHz"];
freq = ["1MHz", "25MHz", "27.5MHz"];
ch1 = 3;        % Numerator opacity
ch2 = 1;        % Denominator opacity

%Import the data from saved files
data = importdata('norm_cal_fin2.mat');
Rcc = data.cellsRN; % Rcc -> Real (in-phase), cell, calcified
Icc = data.cellsIN;
Rbc = data.beadsRN;
Ibc = data.beadsIN;
clear data
data = importdata('norm_decal_fin2.mat');
Rcd = data.cellsRN;
Icd = data.cellsIN;
Rbd = data.beadsRN; % Ibd -> Imaginary (quadrature), bead, decalcifed
Ibd = data.beadsIN;

%% Plot different sets
figure
plot(Rcc(:,ch2),Rcc(:,ch1),'o','LineWidth',1)
hold on
```

```
plot(Rcd(:,ch2),Rcd(:,ch1),'o','LineWidth',1)

xlabel(['Normalized peak height at: ', char(freq(ch2))])
ylabel(['Normalized peak height at: ', char(freq(ch1))])
title('In-phase peak heights')
set(gca,'FontSize',14)
grid on
legend('Cal','Decal','Location','northwest')

figure
plot(Icc(:,ch2),Icc(:,ch1),'.','LineWidth',1)
hold on
plot(Icd(:,ch2),Icd(:,ch1),'.','LineWidth',1)

title('Quadrature peak heights')
xlabel(['Normalized peak height at: ', char(freq(ch2))])
ylabel(['Normalized peak height at: ', char(freq(ch1))])
set(gca,'FontSize',14)
grid on
legend('Cal','Decal','Location','northwest')

%% Analyse data
% Calculate the in-phase and real opacities of the beads, calcified- and decalcified cells
OPIc = Icc(:,ch1)./Icc(:,ch2);
OPId = Icd(:,ch1)./Icd(:,ch2);
OPIbc = Ibc(:,ch1)./Ibc(:,ch2);
OPIbd = Ibd(:,ch1)./Ibd(:,ch2);

OPRc = Rcc(:,ch1)./Rcc(:,ch2);
OPRd = Rcd(:,ch1)./Rcd(:,ch2);
OPRbc = Rbc(:,ch1)./Rbc(:,ch2);
OPRbd = Rbd(:,ch1)./Rbd(:,ch2);

% Find boundaries of histograms
minI = min([OPIc;OPId;OPIbc;OPIbd]);
maxI = max([OPIc;OPId;OPIbc;OPIbd]);
minR = min([OPRc;OPRd;OPRbc;OPRbd]);
maxR = max([OPRc;OPRd;OPRbc;OPRbd]);

%% Plots analysis
% Histogram on opacity using probability normalization to compensate for
% different sample amounts between measurement sets
figure()
subplot(2,1,1);
edge = linspace(minR,maxR,100); %Separate the opacities in 100 histogram bins
histogram(OPRc,edge,'Normalization','probability')
hold on
histogram(OPRd,edge,'Normalization','probability')
histogram(OPRbc,edge,'Normalization','probability')
histogram(OPRbd,edge,'Normalization','probability')
xlabel('Opacity In-phase signal')
ylabel('Probability')
set(gca,'FontSize',14)
grid on
legend('Cal','Decal','Beads cal','Beads decal','Location','northwest')

subplot(2,1,2)
edge = linspace(minI,maxI,100);
histogram(OPIc,edge,'Normalization','probability')
hold on
histogram(OPId,edge,'Normalization','probability')
histogram(OPIbc,edge,'Normalization','probability')
histogram(OPIbd,edge,'Normalization','probability')
xlabel('Opacity Quadrature signal')
ylabel('Probability')
set(gca,'FontSize',14)
```

```
grid on
legend('Cal','Decal','Beads cal','Beads decal','Location','northwest')

% Plot the peak height comparison with the two frequencies used for opacity
figure()
plot(Icc(:,ch2),Icc(:,ch1),'.b')
hold on
plot(Ibc(:,ch2),Ibc(:,ch1),'.y')
plot(Icd(:,ch2),Icd(:,ch1),'.r')
plot(Ibd(:,ch2),Ibd(:,ch1),'.m')
legend('Calcified cells','Beads of calcified','Decalcified cells','Beads of decalcified','Location','northwest
xlabel(['Normalized quadrature peak height at: ', char(freq(ch2))]);
ylabel(['Normalized quadrature peak height at: ', char(freq(ch1))]);
title('Comparison of normalized peaks')
set(gca,'FontSize',12)
```