Contents

1. GENERAL REMARKS & HAZARDS

General remarks

Note 1: This manual may contain errors. Individual instruments may differ from the drawings and photos. You can e-mail spotted errors to info@cytobuoy.com.

Note 2: Always power down the instrument before (dis)connecting or (dis)assembling components. Never apply force. Contact us in case of doubt.

Hazards

General

This CytoSense shall be serviced by specially trained staff only.

Laser

This CytoSense contains a CLASS 3B laser emitting visible light. The laser is fully integrated into the instrument for operator safety. The beam will not be exposed, even when taking the instrument out of the hull. However, take all necessary precautions before opening the optical unit inside this CytoSense.

WARNING: Never look into the laser beam as this can cause serious eye damage and possible blindness.

Electrical

High voltage! The photomultipliers (PMTs) inside the optical unit are powered by a low power high voltage (HV) supply, max. 3000 V at 0.2 mA. Although properly insulated, care must be taken to ensure operator safety. Avoid any direct contact when supply voltage is applied. Earth/ground-devices must have all touchable metal parts and the metal case connected to the earth/ground contact. The CytoSense has proper earth/ground connection through its power supply, which must be connected to a wall receptable with proper earth/ ground contact. When using another power supply, check for proper earth/ground connection.

CytoSub: When using the CytoSub with a battery pack there is no earth/ground connection through a cable. This is not a problem in submerged operation, but in case such a combination is used in the laboratory an earth/ground connection of the housing should be installed by a qualified technician.

Poison and biohazard

This CytoSense handles biological material, it is normal practice to add poisonous preservatives to the working fluid to prevent bacterial growth in the filters and tubing. High bacterial concentrations are not impossible depending on the analysed sample and dose of preservative.

WARNING: The fluids and filters must be treated as dangerous contaminated material and should be disposed accordingly.

2. SHIPMENT CONTENTS

Upon arrival

Check the contents of the shipment for the presence (and any visible damage of):

- The flightcase (figure 2.1)
- CytoSense instrument packed in an aluminium frame
- Laptop computer with power supply and documentation
- USB cable to connect the laptop to the CytoSense
- Power supply for the CytoSense
- Toolbox including:
    - 1 terminal pocket screwdriver
    - Phillips/crosspoint screwdriver, No. 0x3in
    - Phillips/crosspoint screwdriver, No. 1x3in
    - Phillips/crosspoint screwdriver, No. 2x4in
    - Ergonomic Hexagon ball driver, 3 mm ball
    - L-Shape hexagon ball driver, 1.5 mm A/F
    - L-Shape hexagon ball driver, 2 mm A/F
    - L-Shape hexagon ball driver, 2.5 mm A/F
    - L-Shape hexagon ball driver, 4 mm A/F
    - L-Shape hexagon ball driver, 6 mm A/F
    - Versatile insulated trimmer tool
    - Precision slotted screwdriver 1.50x40
    - Torx(R) screwdriver, Tx8
    - Torx(R) screwdriver, Tx10

If there is any visible damage or if there is anything missing, please contact CytoBuoy as soon as possible.

Figure 2.1
Reusable CytoSense flight case.

3. UNPACKING THE CYTOSENSE

To inspect the CytoSense internally after transport, the CytoSense needs to be opened. See figure 3.1 for reference.

1. Remove the 8 screws at the bottom of the instrument that hold the hull in place.
2. Push the fluidic connectors down and fasten them on the bottom plate of the instrument.
3. Gently and slowly pull the instrument vertically upwards out of the cylinder.
   WARNING: Keep the instrument perfectly vertical during sliding it out of the cylinder to prevent damage to the instrument - CytoBuoy strongly advice to do this with two persons for visual control.
4. Place the instrument on a firm, steady, clean and flat surface.

Note: We strongly advise when the instrument is in use to place the instrument in its hull. The hull protects the instrument and makes sure the optical detectors do not detect stray light.

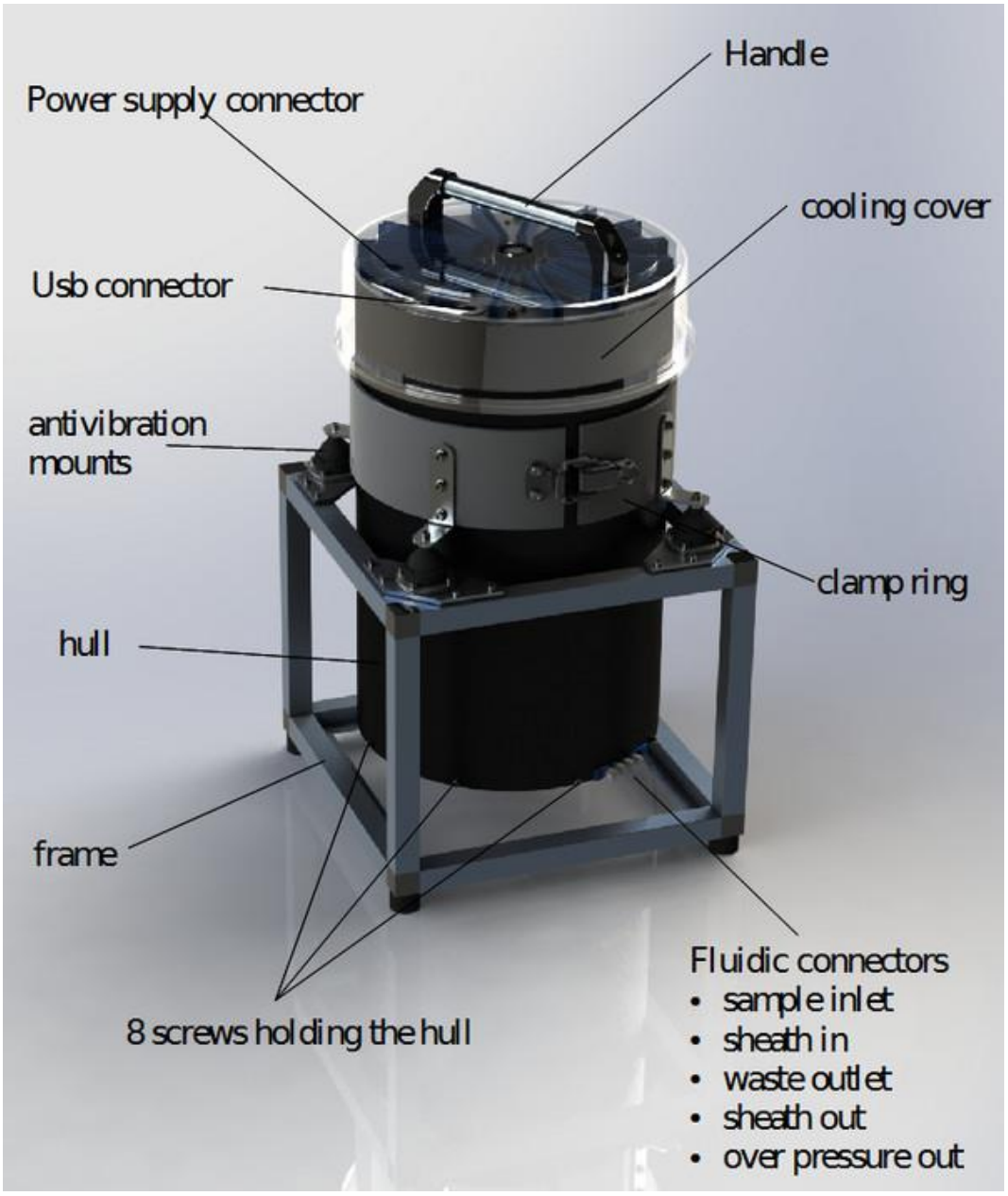


Figure 3.1 CytoSense in shipboard suspension.

4. PREPARING FOR FIRST OPERATION

Before the instrument can be used its internal battery must be connected. It is important because the internal battery protects the electronics of the instrument and it serves as a buffer in the case there are small drops or peaks in the power supplied.

Connecting the internal battery

1. Take the CytoSense out of its hull (see chapter 3).
2. The internal battery was disconnected for transport. Connect the internal battery by closing the fuse holder with the fuse in place (see figure 4.1).
3. Choose to place the CytoSense back in its housing or leave it out. It is advised to leave the CytoSense out of its hull for this **first** operation. This allows you to observe the operation of the sample pump and injector and check the location and operation of various components.
4. Mount the cooling cover (figure 3.1).

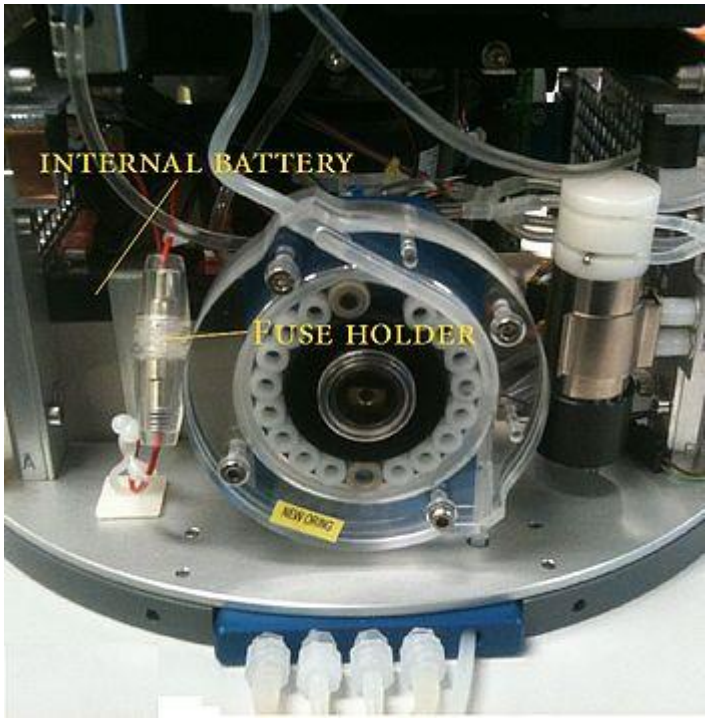


Figure 4.1 Connecting the internal battery.

Note 1: For routine operation it is strongly advised to put the CytoSense in its hull. The hull prevents the instrument from stray light, dust and mechanical damage and is important for cooling.

Note 2: When you put the CytoSense back into the hull: keep the instrument perfectly vertical during sliding it into the cylinder to prevent damage to the instrument - CytoBuoy strongly advises to do this with two persons for visual control.

Note 3: If the CytoSense fluidic connector are the same as shown in figure 4.1, make sure the luer holder is folded inwards.

Preparing the fluidics

Remove the small plugs from the sample input line, waste outlet tube and pressure relief tube at the bottom of the CytoSense (see figure 3.1). You may cut a piece from the sample and/or waste line to shorten it to the desired length for your purposes.

Note 4: If you lengthen or shorten the sample tubing, make sure you adjust the flushing times in CytoUSB accordingly.

Connecting the power supply

The power supply accepts 100-240 volt AC (47-63Hz). The CytoSense power connector is located on the top of the instrument. Make sure the fuse is placed in the fuse holder and the fuse holder is closed before connecting the power cord to the instrument.

Connecting the instrument to the computer

Before you can control the instrument, you will need to install CytoUSB and the CytoDriver programs. These can be downloaded from www.cytobuoy.com (requires login).

1. Unpack the computer and place it on a flat and stable table, close to the CytoSense. The instrument is controlled via a USB (Universal Serial Bus) interface.
2. Plug the flat usb connector into any usb ports of the computer and connect the other side to the CytoSense. The USB connector is located on the top of the instrument. The CytoSense is fully computer controlled and has no mechanical controls.
3. Start up the CytoUSB program to activate the CytoSense. See the CytoUSB manual for further instructions.

5. PRINCIPLE OF OPERATION

Flow cytometers count and analyse individual particles in a fluid. The particle suspension (sample) is injected into a particle free carrying fluid (sheath) that narrows down the suspension into a very thin line of fluid in which the particles are gently stretched out into a single file and aligned along the path of flow (figure 5.1). This 'line of particles' is moving at a fixed high speed exactly through the middle of a sharply focused laser beam.

From each passing particle the scattered laser light is measured at two angles as well as several colors of laser induced fluorescence emitted by chlorophyll and other pigments present in algal cells.

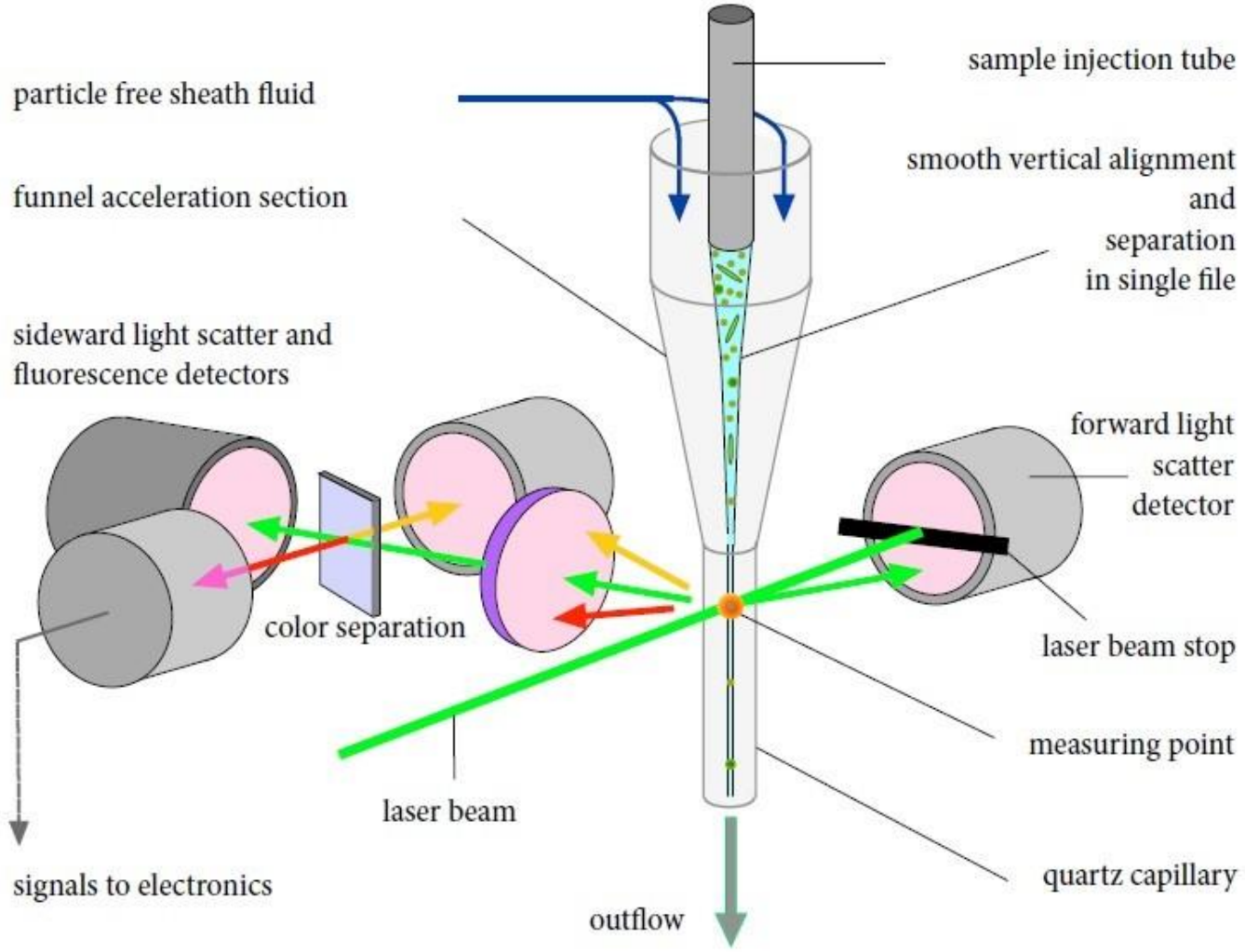


Figure 5.1 Analysis principle of flow cytometry

The sample flows into the relatively wide and funnel shaped injector through a hollow needle while being surrounded by a much larger amount of co-injected particle free sheath fluid.

When the sample approaches the narrow flow channel of the cuvette, the fluid cross section of both the sheath fluid and the suspension stream sharply decreases, whereas their relative position remains (nonmixing laminar flow conditions). As a consequence the flow velocity increases sharply and the particles get lined up at large distances from each other.

The final velocity of the line of particles passing through the laser beam is fixed and governed by the sheath fluid flow rate only (not by the sample flow rate). The default sheath velocity is ca. 2 m/s. The sample pump flow rate does not influence the velocity in the cuvette significantly; at maximum sampling rate the cuvette flow velocity is increased by no more than 1.5%.

The diameter of the sample stream is proportional to sample flow rate (the square root of). This suspensions stream intersects the laser beam which is directed straight through the flow cell at an intersection point. Particles flowing through this point scatter the laser light and may emit fluorescent light, which is detected by the detection system.

The detectors convert the light pulses into electronic pulses. These pulses are subsequently digitized and processed. The optical properties such as light scattering and fluorescence of about 1000 or more particles per second can be measured. The resulting data is processed and analyzed using a computer, the measured data can also be used in real time in some instruments to activate a downstream device such as an imaging device (optional) or a sorting unit (optional) to sort out individual particles of interest from the suspension stream.

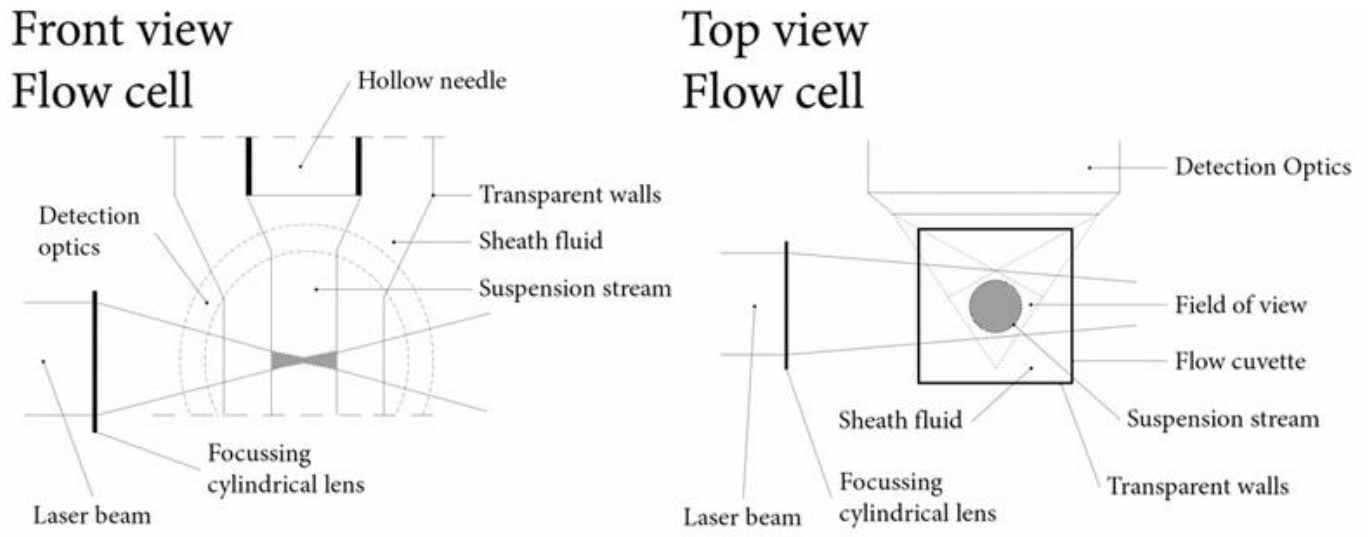


Figure 5.2 Sensing zone inside the flow cell

Figure 5.2 shows the front (left panel) and top (right panel) view of the flow cell. The flow cell contains the suspension stream in the middle surrounded by the sheath fluid. The detection optics, depicted schematically, are placed behind the flow cell, having a 'field of view' from which the emitted light can be detected. Only particles inside this 'field of view' can be detected by CytoSense.

This intersection (sensing) volume ('field of view') should be as small as possible to keep the signal to noise ratio maximal, but large enough to contain the large particles. The light intensity should be uniform and as high as possible to enable detection of small particles. Getting high light intensity from a relatively small laser requires a narrow beam. However, the Gaussian distribution of the light intensity over the width requires a spot many times larger than the diameter of the suspension stream.

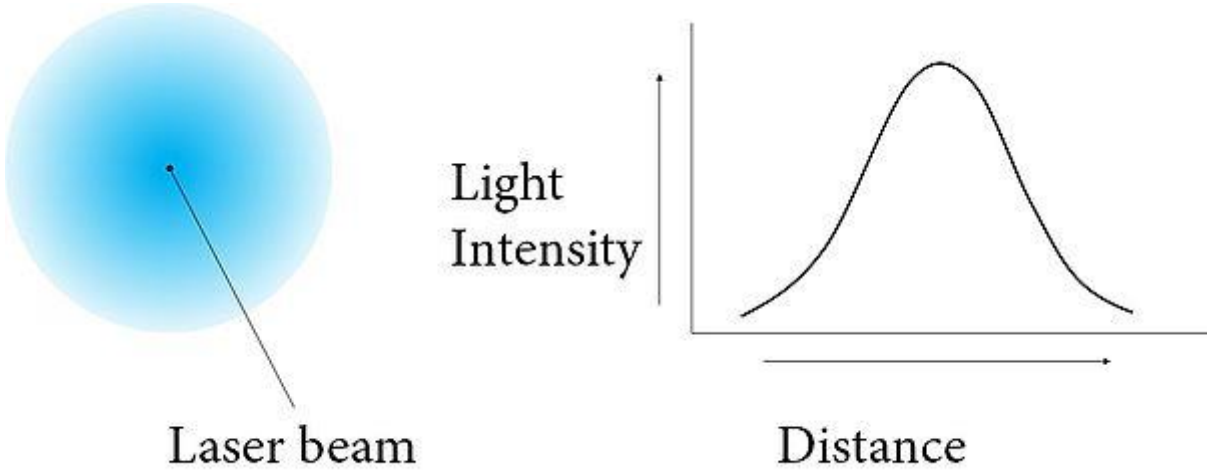
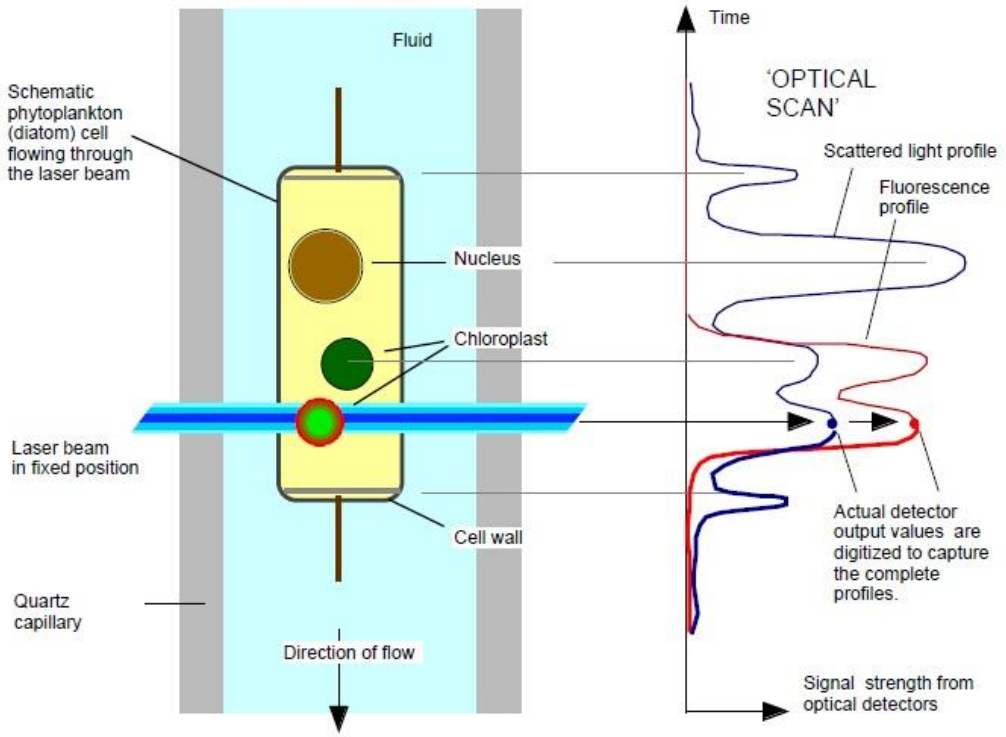


Figure 5.3 Simulation of a Gaussian beam cross-section proportional to the light intensity (left). The corresponding intensity as a function of the distance from the middle (right).

Particles flowing through the intersection volume outside the middle will experience a much lower light intensity. It is therefore crucial that the sample stream intersects with the center of the laser beam! A large sensing zone as used in CytoSense would require a large beam width to yield a low variation in light intensity which would unfortunately also mean a low light intensity level, whereas a large part of the light hits the cuvette walls causing unwanted background scatter. CytoSense uses a double beam solution to circumvent these drawbacks. This double beam feature is also used for the so-called "curvature" option. The beam shaping is explained in more detail in the paragraph on Curvature (see chapter 7).

Figure 5.4
CytoSense scanning principle



The CytoSense is a fixed beam scanning flow cytometer, which yields 1-dimensional profiles obtained from particles passing at constant velocity through the laser beam.

The amplitude, length and shape of these signal courses are governed by the morphology of the particle: its size and the distribution of its 'body parts' including chloroplasts along its length axis. An example of such a set of scanned profiles of one individual particle is depicted in Figure 5.4figure 5.5, and the CytoClus manual).

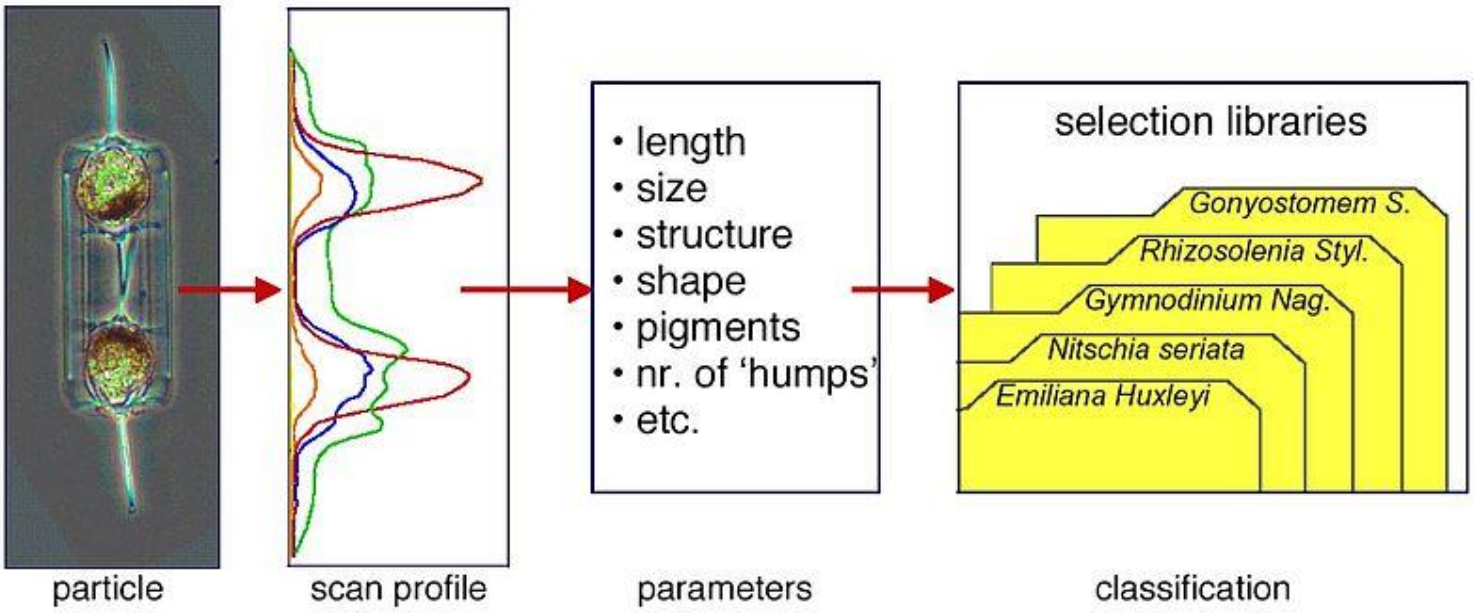


Figure 5.5 Data processing

6. MAIN COMPONENTS OF THE FLOW CYTOMETER

A scheme of the main components of the CytoSense flow cytometer are shown in figure 6.1.

The fluidic section consists of a sample pump taking in the required amount of sample fluid and a sheath pump that carries the particles inside the accelerated sample stream with the required speed and precision through the right spot ('field of view', figure 5.2).

The optical section consists of a light excitation section with a laser and beam focusing optics to provide the required level of illumination at the 'field of view', and a detection section with light collection optics, color separation dichroic mirrors and detectors to measure the various light scatter and fluorescence photon fluxes emitted by the particles while they pass the sensing zone.

The electronics section consist of a power interface, a data processing section and a USB interface. The power interface converts the computer commands to power the various electrically driven components of the fluidics and optical section at the right voltages and times. The data processing section converts the output signals of the photodetectors to a data stream ready to be transferred to the computer. The USB interface controls the USB communication with the computer.

On the computer there are two programs installed, one for operating the machine and conducting the measurements with the flow cytometer (CytoUSB) and the other for analysis of the data (CytoClus).

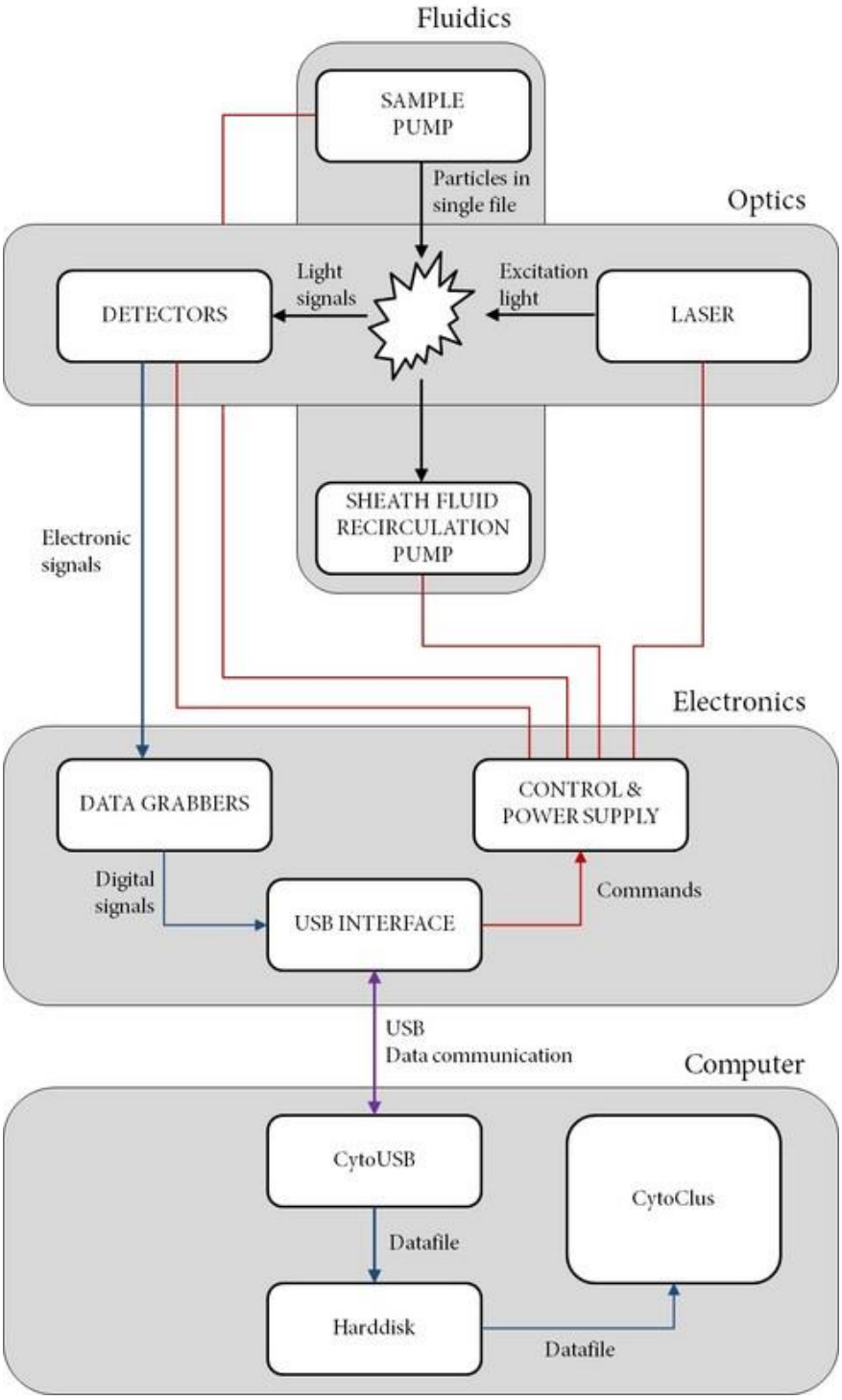


Figure 6.1 Scheme of the main CytoSense components

7. OPTICAL SYSTEM

The laser beam is shaped into a flat beam of 5 micrometers high and about 300 micrometers wide before passing through the cuvette (see figures 7.1, 7.2 and 7.3

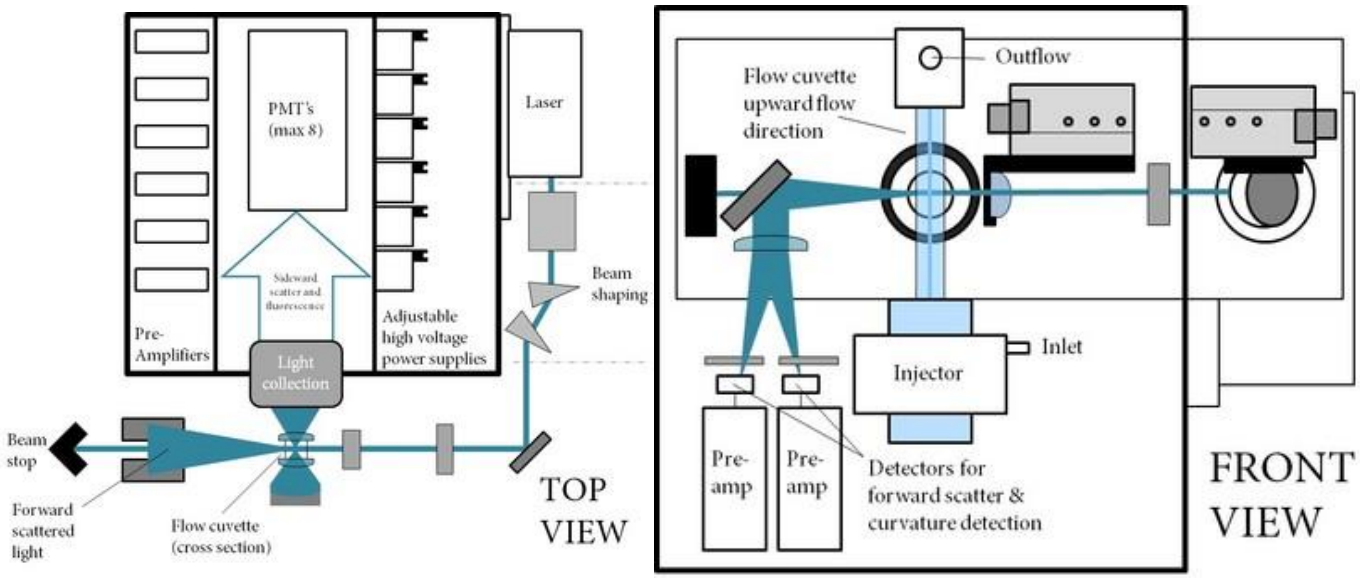


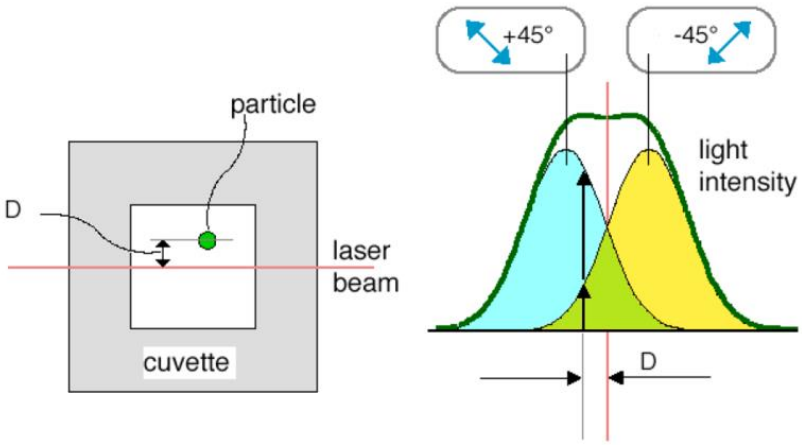
Figure 7.1 et 7.2 Schematic views of the optical system

Light emerging at 90 degrees to the laser beam is collected by an objective and directed by a set of mirrors through another series of mirrors and filters onto a series of detectors. Light at wavelengths longer than the laser wavelength originates from fluorescence in the particle, and it is much weaker than the forward scattered light. Therefore the detectors used here are PhotoMultiplier Tubes (PMTs).

The wavelength selection for all the PMTs is factory preselected for the intended research of a customer. If the requirements change the dichroic mirrors and optical filters (long pass or band pass) in front of the PMTs can be changed by CytoBuoy. The spectra of the dichroics used can be found here.

Curvature

The CytoSense uses a double laser beam solution to obtain a flat light distribution over the detectors sensing zone (see chapter 5), more efficient as compared to the standard Gaussian (bell shaped) distribution. This is achieved by the superposition of two Gaussian beams with parallel beam paths and partly overlapping light distributions. Interference is prevented by arranging the polarization states of these beams perpendicular to each other.figure 7.3.



Chaetoceros curvisetus, also Tabellaria sp. distinct variations in the forward scatter polarization ration occur. Therefore we call it 'curvature' signals. It is not imaging but it yields extra two-dimensional information out off a low cost and data extensive extra channel.

In CytoClus, the signals from both left and right hand forward scatter detectors are summed for 'forward scatter' and subtracted for the 'curvature' parameter.

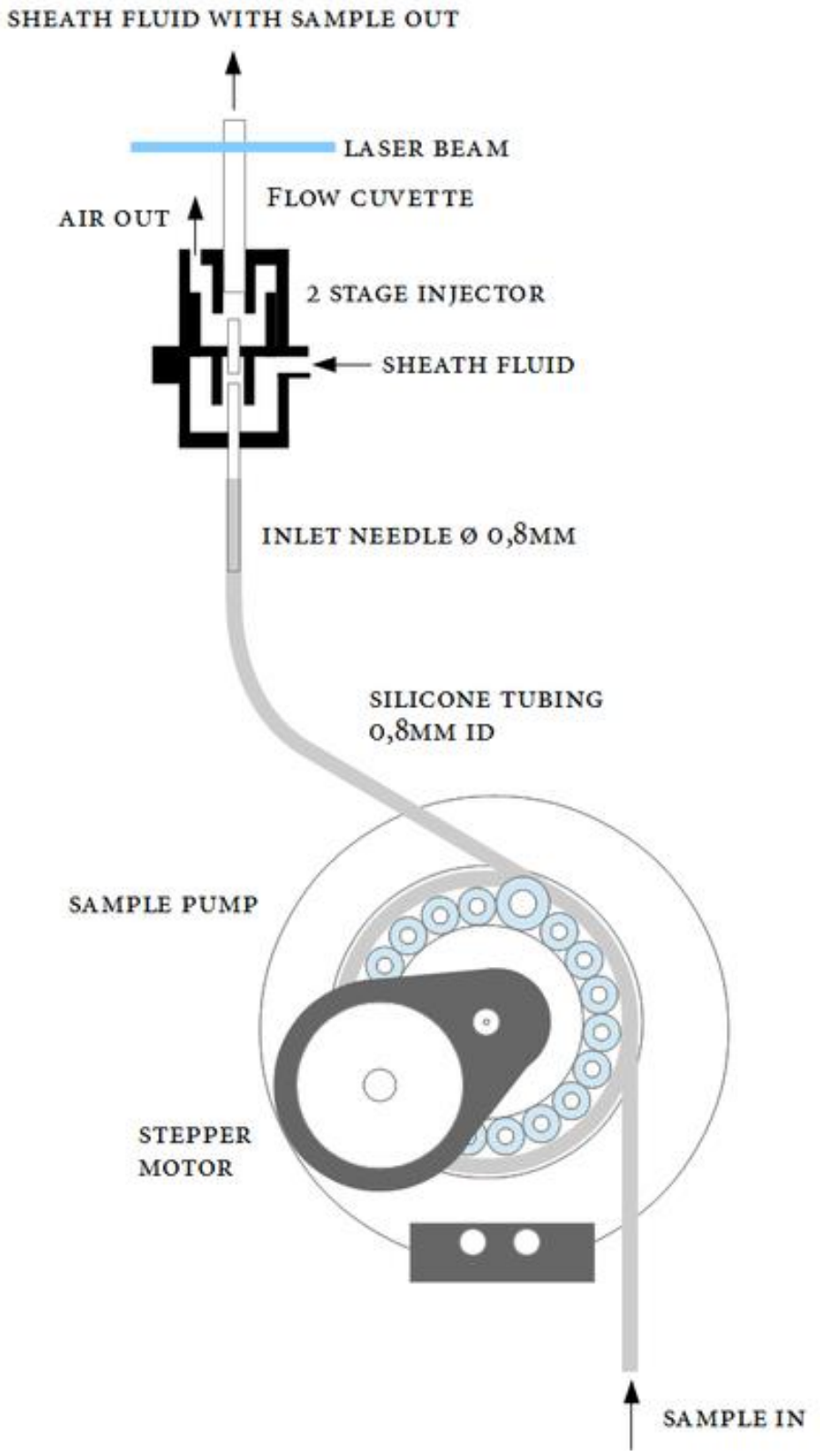
Figure 7.3 Off-center particle trajectory in flow cell (left); corresponding illumination by the two beams (right)

8. FLUIDIC SYSTEM

The sample fluid is pumped into the CytoSense by a peristaltic sample pump through silicon tubing (figure 8.1)

A basic diagram of the sheath fluid re-circulation system is shown in figure 8.2

Figure 8.1 Sample input section



Note: Therefore: add biocide frequently, more frequently if the sampling frequency is high.

Not all biocides are safe for the materials used in the cytometer fluidics (quartz, silicone tubing, nylon connectors, stainless steel, filter housings). The sheath system comes filled by default with Proclin 950. To mix the biocide properly in the system, run the sheath pump for 5 minutes.

See chapter 15. Or you can check the pressure sensor read outs in CytoUSB.

Matching sheath fluid and sample fluid

The CytoSense comes filled with non-saline distilled water. For critical applications we advise that the density and refractive index of the sample fluid, which forms the core in the cuvette, and the sheath fluid that surrounds the core should be matched as closely as possible. If the sample is heavier than the sheath the effect of this mismatch in density can be that the core in the cuvette may become less stable in its position. This can happen if a seawater sample is run with distilled water as sheath fluid in the filters. As the flow in the cuvette is upward this will not happen if the sample fluid is lighter as is the case with a freshwater sample and salt water in the filters. A mismatch of refractive index may cause a low level of forward scattering by the core itself, additional to the scattering by the particles. If 'forward scattering' is used as the trigger source, this may cause some degree of triggering on "false" particles, which shows up as a "baseline" in the dotplot of forward scatter vs length (see chapter 12). Procedures to change sheath fluid from fresh to saline or vice versa are described in chapter 16.

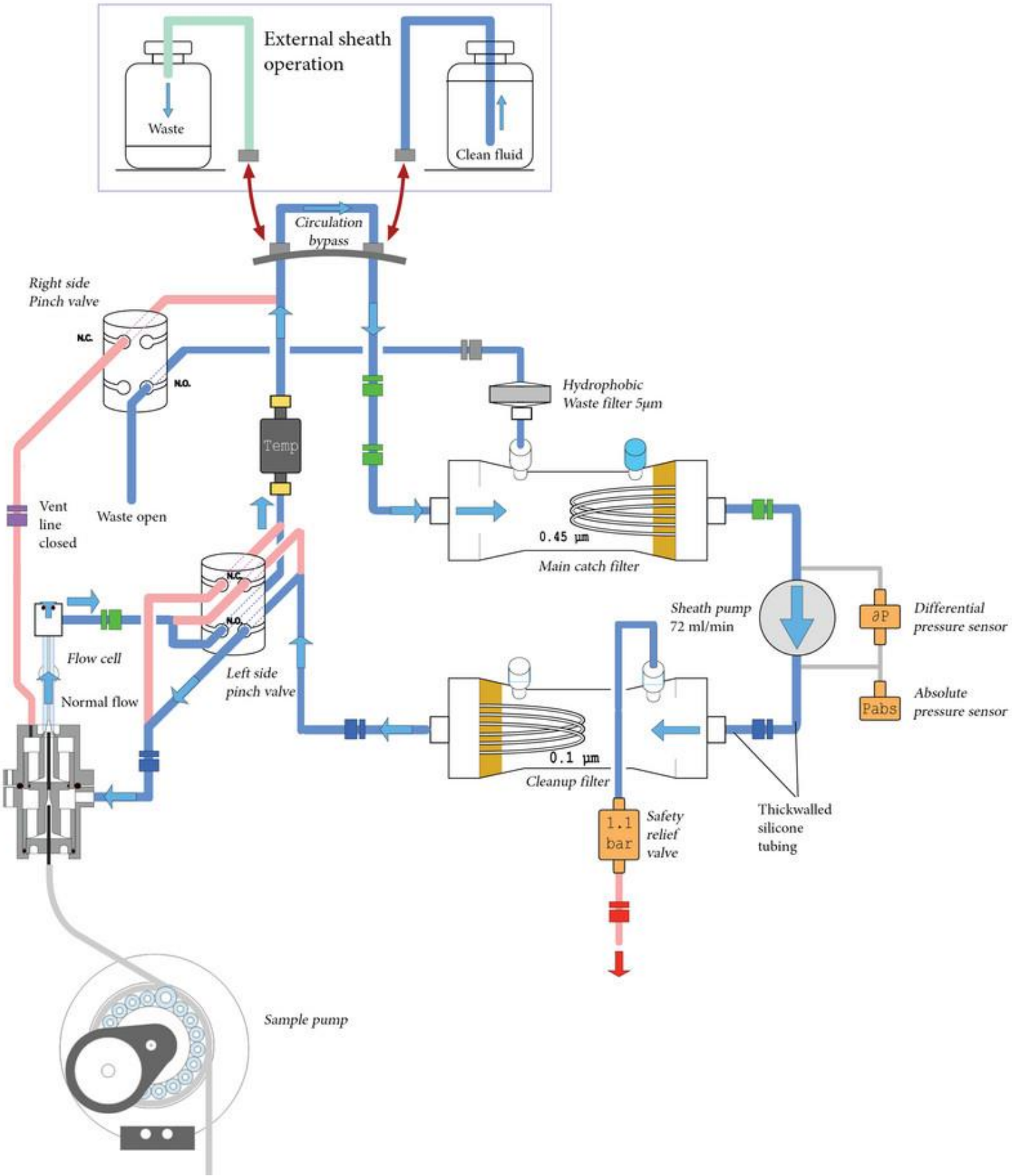


Figure 8.2 Fluidic system

9. ELECTRONICS

The grabber PCB converts the output signals of the detectors to a data stream ready to be transferred to the computer. The USB PCB handles the communication with the computer.

The optional Imaging In Flow electronics houses a digital signal processor that handles the data in real-time. When a particle matches certain criteria, the Imaging-in-flow system will take a photograph of the particle.

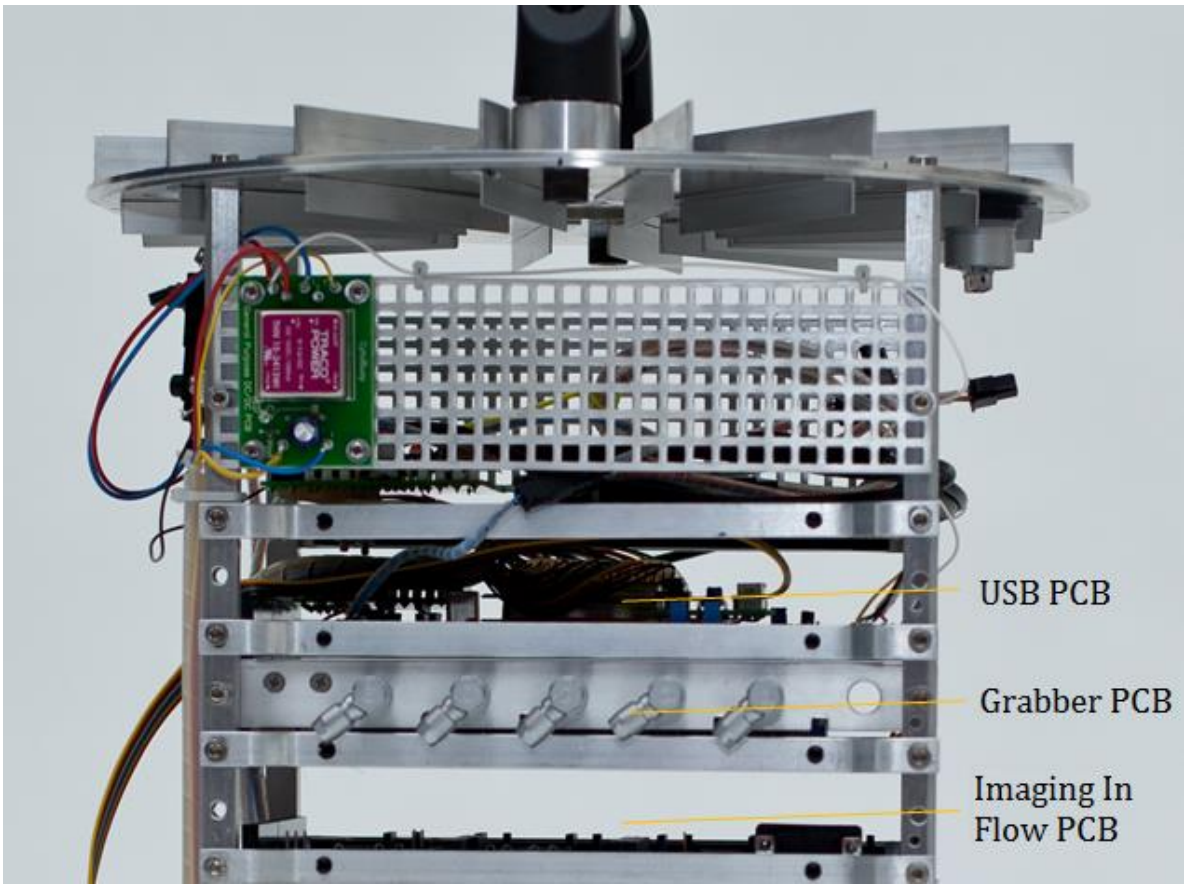


Figure 9.1 CytoSense electronics

Optical Unit

The signals from the detectors are amplified for further processing in the electronics. To avoid noise and interference the amplifiers are located close to the detectors in the optical unit, in individual metal electromagnetic shielding casings.

Electronic Unit

The pre-amplified signals are processed by individual grabber-circuits (channels) located on the grabber PCB. One grabber PCB maximally contains 6 channels. If more than 6 detectors are present in the CytoSense, a second grabber PCB is installed.

The order of the connectors on the grabber boards is factory determined and corresponds to the software. Colored tie-wraps are attached to the plugs. When loosening these connectors for monitoring the signals on an oscilloscope, be sure to put them back in the same order.

10. INSTALLATION & OPERATION

Installation

The standard CytoSense is not protected against rain, a full waterproof housing is required for outdoor or submersed use. For surface or shallow depth applications a light weight pressure housing can be ordered from CytoBuoy b.v. For use in standard depths over the photonic zone (max. 200 meter depth) the standard CytoSub can be used. For moored use the CytoBuoy should be used.

Sample inlet: In field installations for *in situ* measurements sample has to be transported to this tube in some manner. Various sampling accessories can be ordered.

Laboratory installation

Install the CytoSense in a convenient place so that the sample inlet tube can be easily placed into the sample containers. Provide some container to catch the outflow of waste. As the sheath fluid in this CytoSense is recirculated, the waste volume is about the same as the sample volume that is taken in. Note that the speed in the sample line is only about 1 cm/sec.

Note: To reduce flushing times, keep the sample tube short.

Field installation with high-pressure sample loop (CytoSub)

The pump to circulate the water through the sample loop should be self priming and suitable for dirty water. Good pumps for this purpose are flexible impeller pumps and membrane pumps with large valve flaps. Height above water of the installation should be as low as practical since the pressure in the sample drops with height and that can cause dissolved air to form bubbles.

Operation

Although the operation is controlled by computer programs the following advice should be observed :

WARNING: Avoid pumping in air. Do not run the CytoSense with the sample inlet dry.

The control program (CytoUSB) flushes the sample inlet tube with clean sheath fluid after a measurement. After using the machine, switch the power off and clean the outside of the sample inlet tube.

When using the CytoSense to analyze sea water, the sheath system should be filled also with water of similar salinity for best results. However, when salt water dries out the salt will crystallize inside the tubing and pump gears, inhibiting proper operation of the sample pump and sheath pump.

When the machine is not used on a daily basis

1. Close the inlet tube with a stopper and/or to flush the sample line with tap water after measurements.
2. Run both the sheath pump and the sample pump for ca. 10 minutes at least once a week. In case the instrument is used with salt (sheath) water, it is advised to exchange all fluids with fresh water. In that case the run frequency can be reduced.

Operation procedures

The operation of the CytoSense is organized in three different modes:

1. Interactive mode: each samples is measured manually and each measurement is initiated by the user.
2. Scheduled mode: after the system is configured by the user, on a preset time interval, CytoUSB will perform a measurement without any user input.
3. Manual control: in this mode the user can control the different components (pumps/valves etc.) of the machine.

11. INTERPRETATION OF RESULTS

To make sure that the machine is in perfect order, the user needs to measure a sample with calibration beads. The output of the machine (data) needs to be verified and compared to factory standards.

This chapter will describe how to run a calibration file and compare the data with the factory standard.

Running the first measurement

Before we run the first measurement we need to check the following items:

- Is there any sign of damage to the instrument or the peripherals? (chapter 2)
- Is the machine prepared for use? (chapter 3 and 4)

[6] particles per ml.

To check the status of the instrument, please follow these steps:

1. See figure 3.1. Make sure the sample inlet tube is in the beads solution and the waste outlet is in a waste container. Make sure the over pressure tubing is **NOT** in the waste container. This is because you need to be able to watch the over-pressure outlet during the measurement.

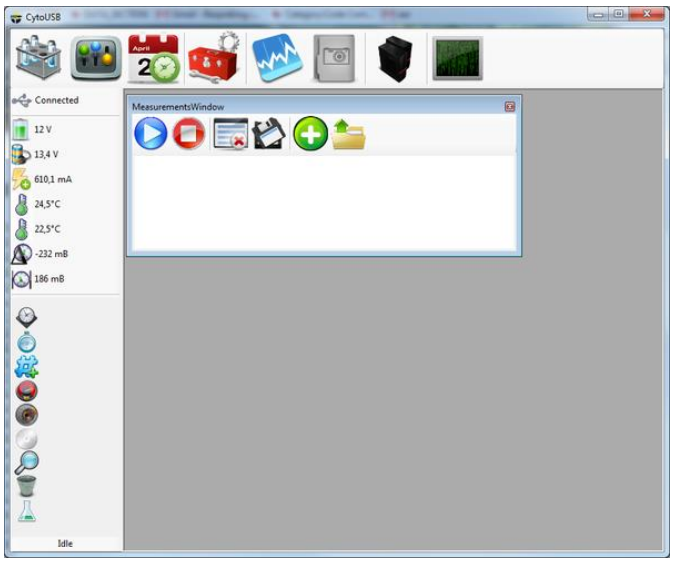
2. Verify that the sheath in and sheath out are connected to each other.

3. Start CytoUSB and inspect the diagnostic parameters.

4. Make sure these parameters are within the limits.

Diagnostic parameter	Safe Range
Internal battery	10 - 13.6 V
Wall power voltage	13 - 13.6 V
Recharge current	0 - 700 mA
System temperature	
Sheath temperature	
Differential pressure	-300 - 0 mbar
Absolute pressure	0.2 - 600 mbar

7. Click the icon  to open the measurements window:

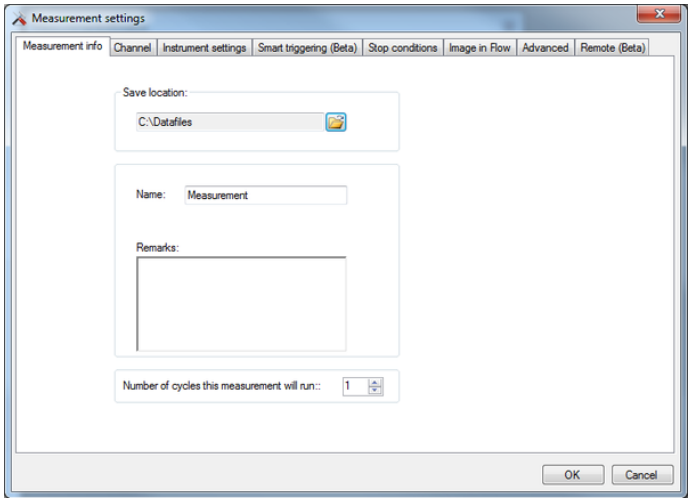


8. Click the icon  to add a measurement.

9. Select location by clicking on the icon  and choosing a folder on your computer.

10. Name the measurement.

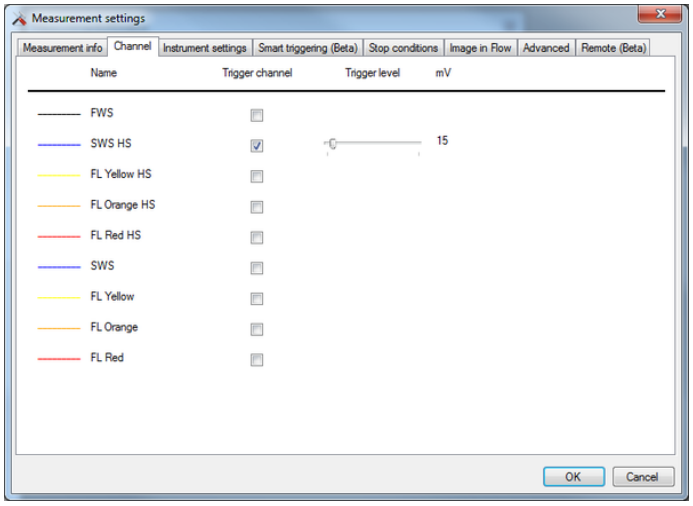
11. Select the "Channel" tab.



12. Select SWS HS as the trigger channel (SWS if your machine does not have a SWS HS).

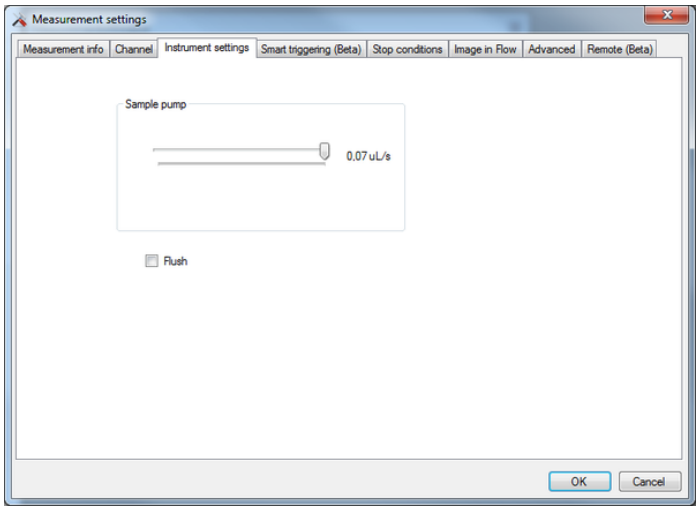
13. Drag the trigger level slider to 15 mV

14. Select the "Instrument settings" tab.



15. Drag the *Sample pump* speed slider to the right, selecting the lowest possible speed.

16. Select the "Stop conditions" tab.

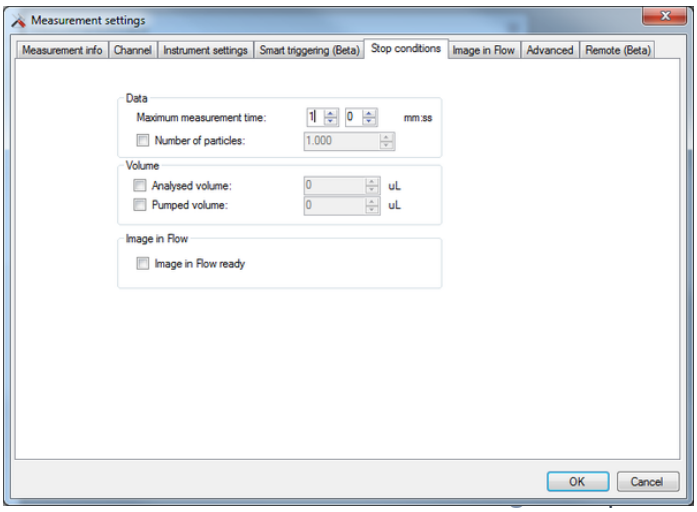


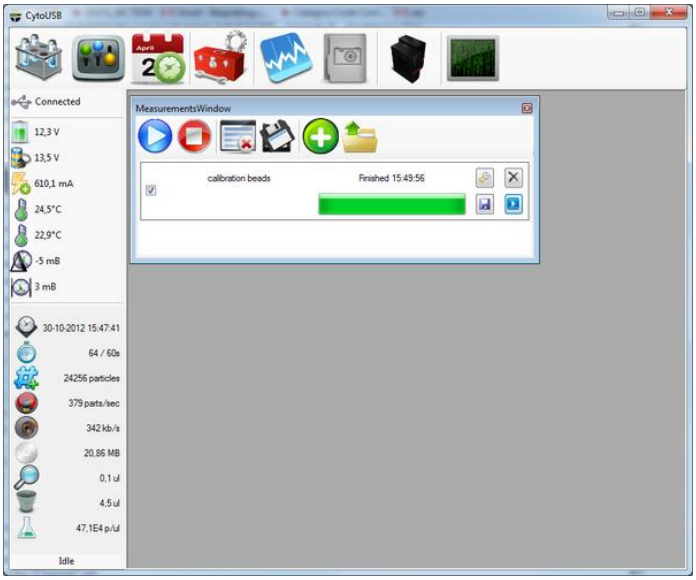
17. Input the *Maximum measurement time*: 1 min 0 s. Make sure all other stop conditions are **NOT** ticked.

18. Click "OK" in the right hand corner of the Measurement settings screen.

19. Click the icon  .

20. The measurement will start, the instrument will begin with a flush and warm up cycle.

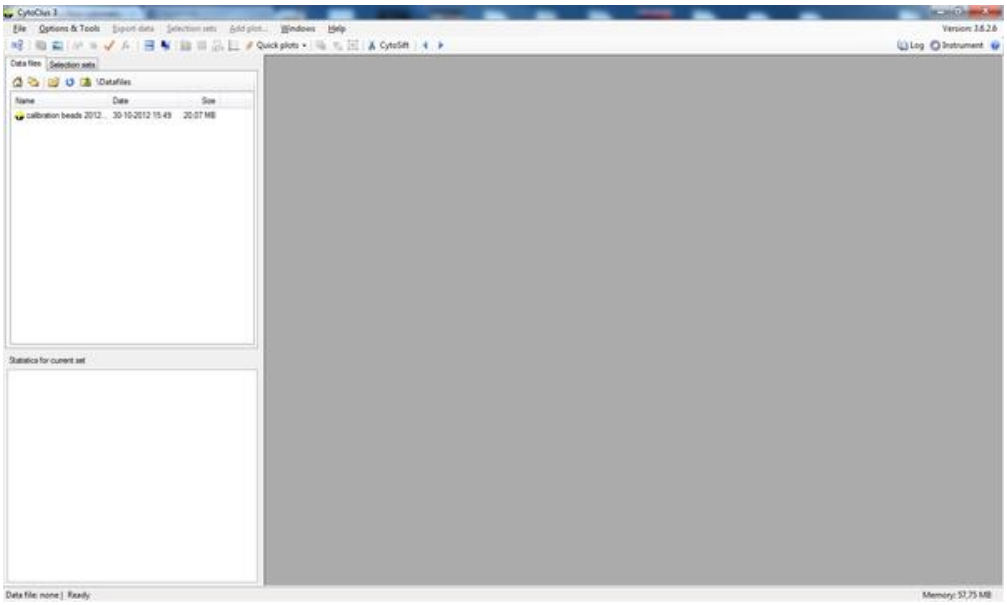




Note: Observe the over-pressure outlet! If it produces fluid it means that there is a blockage in the fluidic system. In this case:

- switch off the machine
- carefully check all the tubing and make sure there are no clamps on the tubing or the tubing is blocked or twisted in any manner.
- check the filters, after long use the filters can clog over time

21. Open CytoClus.



22. Browse to the datafile CytoUSB just created on the left and open the file by left clicking on it.

12. SIGNAL CHECK WITH OSCILLOSCOPE

The analog output of the detectors (raw signal) can be viewed with an oscilloscope. For this purpose a special connection box, the so-called Scope box, is needed (figure 12.1). The Scope box is provided with your machine. For monitoring the raw signal with the oscilloscope, take out the signal plug from the grabber board connector strip (figure 9.1) and place that plug into one of the connectors of the Scope box (arrow 1 in figure 12.1). Now put the corresponding knee-plug from the Scope box in place of the now freed position of that signal plug on the grabber board connector strip (arrow 2 in figure 12.1

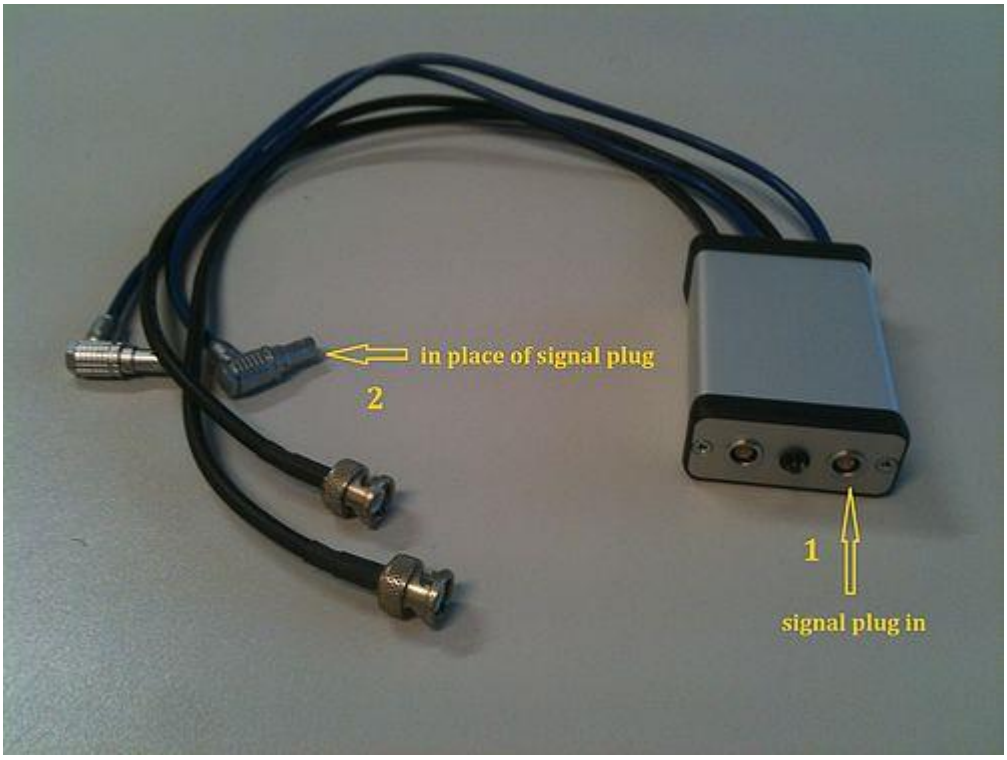


Figure 12.1 A Scope box

13. MAINTENANCE AND STORAGE

The generally advised conditions are:

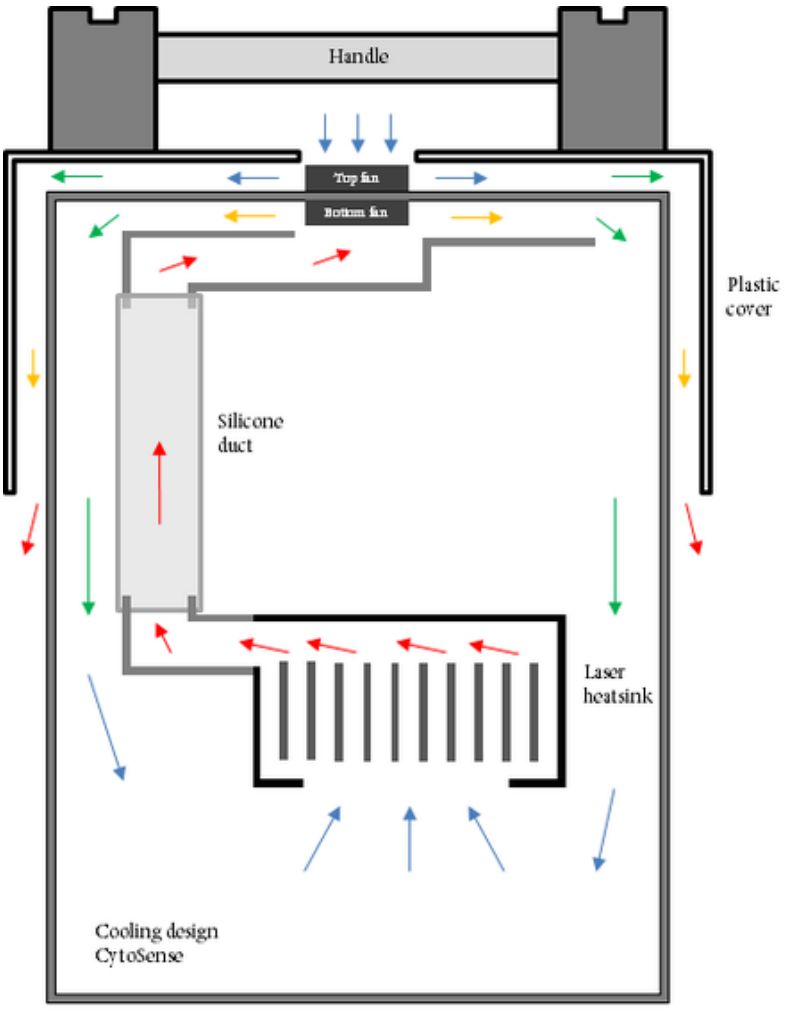
Operating temperature:	
Storage temperature:	
Humidity:	< 80% non-condensing

The advantage of this arrangement is that there is no exchange of air between inside and outside of housing which prevents dust buildup inside instrument.

The internal battery is a, maintenance free, 12 V battery. If the instrument is not used for an extended periods, the battery needs to be charged for about two hours per month. This corresponds to the advised running frequency for the fluidics system.

The CytoSense is made to be as maintenance free as possible. The area of the sheath fluid filters is 5500 cm^2, that is sufficient for about 3000 hours of pumping sample at a particle density that produces a full data set in 10 s, so the filters should last for a year in normal use. In some conditions the filters may clog sooner. In that case you can order a new set of filters from CytoBuoy b.v. or back-flush the filters (see chapter 22).

The system is, in principle, self cleaning but we recommend to run the sheath pump (using "manual control" in CytoUSB) for at least one hour before putting it out of use for longer time, or at least every week when it is used only occasionally. In case you are running marine samples it is advised to flush the silicone sample line (sample pump) and (for CytoSub) the PEEK sample loop (6-port valve and loop filling pump) after each sampling session with fresh water if the instrument will not be used more than 1 day. If the instrument will not be used in more than a month it is advised to exchange the salt sheath fluid for fresh water (do not forget to put some biocide in to prevent bacterial growth). This will protect the sheath pump. Plug the sample inlet, waste outlet and (CytoSub) sample loop lines. If you are not using the instrument for several months it is advised to run the pumps at least 1 hour per month (do not forget to unplug the lines and plug them again afterwards). Do not keep salt water in the instrument without running the system for longer than a week to prevent salt crystal formation that might block the pumps and valve (CytoSub).



Note: Do not drain the fluid from the CytoSense as this will cause debris and/or salt crystals in the tubing and filters which is hard to remove.

Figure 13.1 Cooling design of the CytoSense

14. SHIPPING CYTOSENSE COMPONENTS

Always first switch off all power and remove the fuse (see figure 4.1).

The CytoSense is designed for straightforward (dis)assembly. The optical unit can be slid out the front (figure 14.2); the electronics boards (data grabbers & USB interface board; figure 9.1) slide out to the side. Figure 14.1 shows the layout of the lower part of the CytoSense. The sheath unit can be taken out of the back side; the power interface plus internal battery can be taken out to the left side and the SUB unit (if present) is mounted on a single plate which can be taken out to the right side.

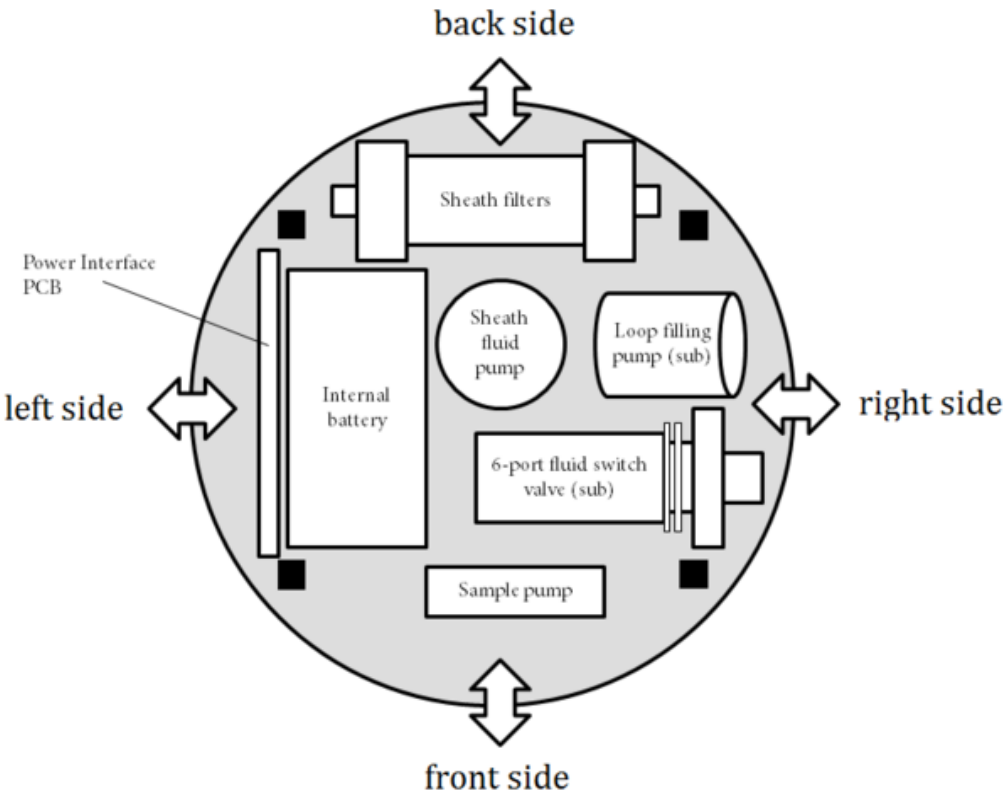


Figure 14.1
Scheme of the lower part of the CytoSense

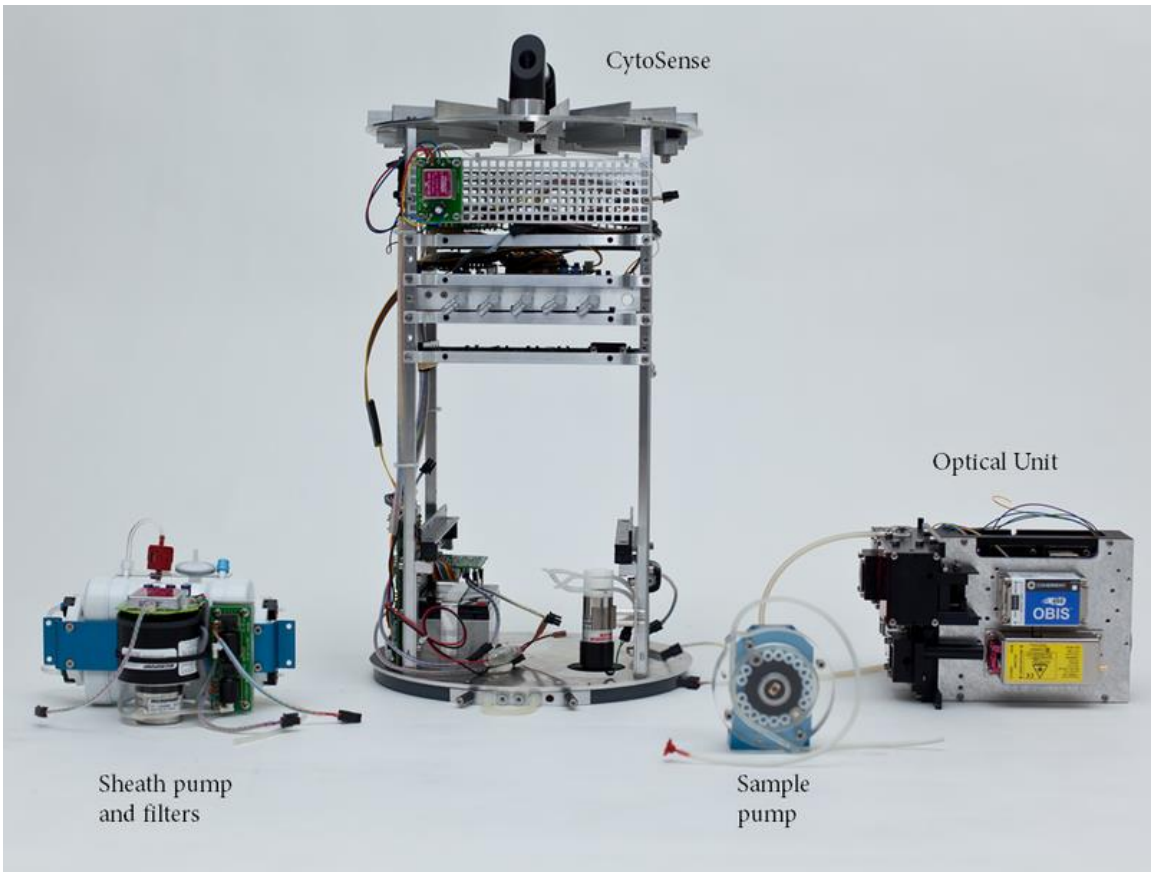


Figure 14.2 CytoSense components

Removing the optical unit

To remove the optical unit, take the following steps:

1. Switch off all the power
    1. disconnect the power cable from the instrument (top side).
    2. remove the fuse from the instrument (located in front of the battery; open the fuse holder and remove the fuse).
    3. Disconnect the USB cable from the machine (top side).
2. Disconnecting the sample line:
    1. Clamp off both sides of the injector to prevent fluid leaking out of the injector when you pull the sample tubing off.
    2. Unplug the sample line from the injector.
    3. Put a paper tissue right under the injector when you gently pull off the sample tubing to catch any water that comes out.
    4. Put a small plugged off piece of sample tubing on the injector needle to close it..
    5. Remove clamps.
3. Unplug the sheath fluid connections on the right side of the instrument and connect both sides of the optical unit and both sides of the sheath unit.
4. Unscrew the plastic air outlet from the back of the optical unit (it has to be removed, or the optical unit won't slide out). **Note:** When you mount this air outlet please make sure the electrical wire bundle falls in the milled out slot to make a tight connection.
5. Disconnect the plastic PMT connector on the back of the optical unit.
6. Disconnect the laser connector on the back of the laser.
7. Disconnect all the detector cables from the grabber board(s).
8. Unscrew the 4 holding screws in the slide bars. To reach the front left screw, it may be helpful to to remove the connector from the power interface PCB.
9. Gently slide out the optical unit towards the front while constantly checking if nothing is blocking the way or still has to be disconnected.
10. For shipment, use the cardboard box and the special plywood plate with flanges that can be obtained from us.

Removing the Sheath pump

The sheath pump can be taken out as well. This is needed if the sheath pump must be repaired. To remove the sheath fluid unit, take the following steps:

1. Switch off all the power
    1. disconnect the power cable from the instrument (top side).
    2. remove the fuse from the instrument (located in front of the battery; open the fuse holder and remove the fuse).
    3. Disconnect the USB cable from the machine (top side).
2. Unplug the sheath fluid connections on the right side of the instrument and connect both sides of the optical unit and both sides of the sheath unit.
3. Slide the waste line upwards through the bottom and plug off.
4. Disconnect the electrical connector (located in front of the internal baterry - next to the sample pump connector).
5. Unscrew the mounting flange from the vertical posts (or from the back flanges in previous models).
6. Now the unit can be taken out as a whole. If this is difficult it might be helpful to unscrew the plastic filter holder bars first and take out the filters.
7. Ship the unit in a sufficiently big cardboard box with protective filling.

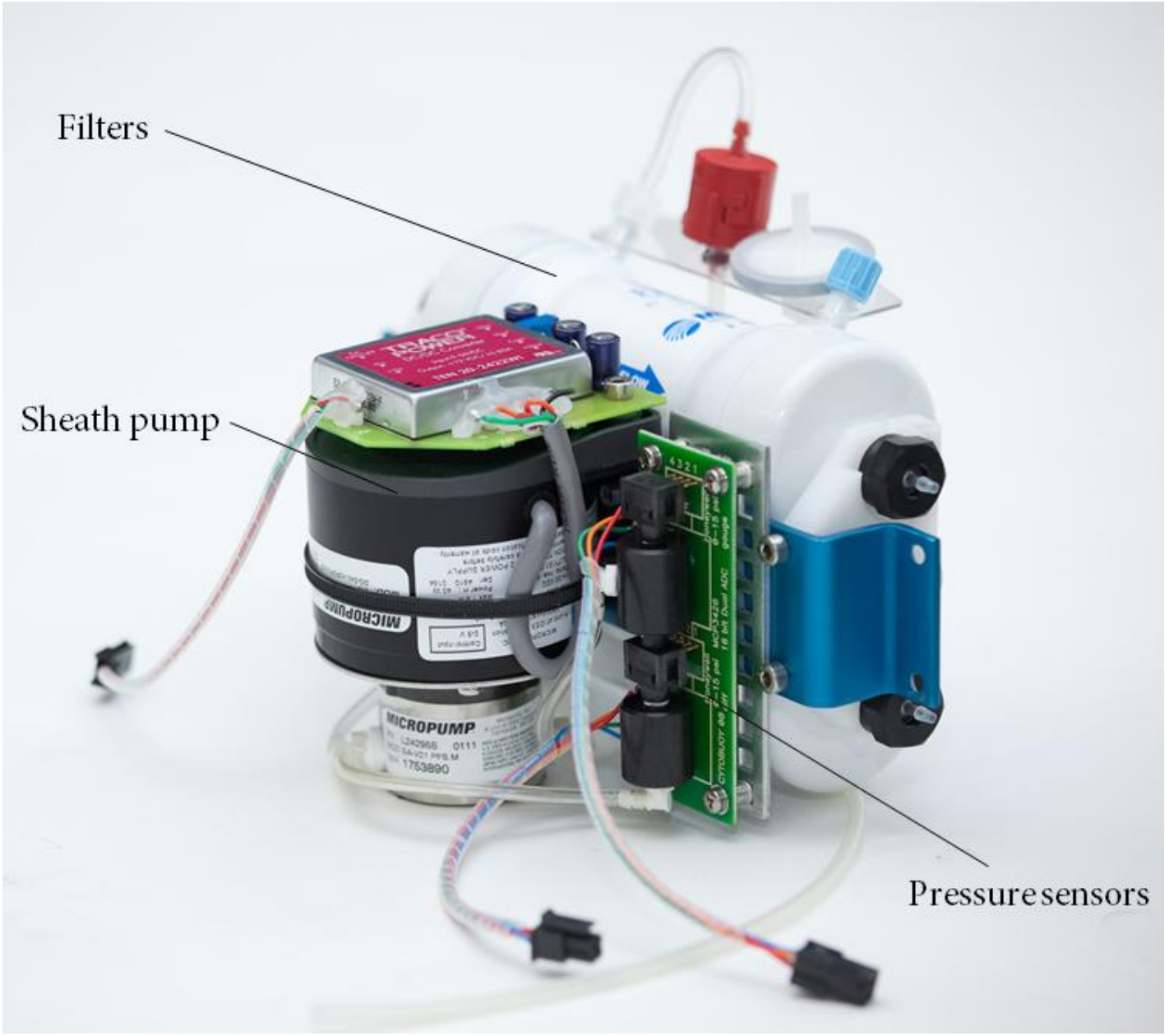


Figure 14.3 Sheath pump unit

15. SHEATH FLUID UNIT: CHANGING FLUID

As this CytoSense handles biological material it is normal practice to add poisonous preservatives to the working fluid to prevent bacterial growth in the filters, but high bacterial concentrations are not impossible.

WARNING: The fluids and the filters must be treated as dangerous contaminated material and should be disposed accordingly.

To change the sheath fluid, please take the following steps (see figure 8.2):

1. Prepare at least 2 liters of clean (fresh or salt) particle free water as new sheath fluid. It does not need to be absolute particle free, but it should at least be filtered. The density should match that of the water in the samples to process.
    1. Add an adequate amount of biocide (e.g. glutaraldehyde, 5% final concentration) to prevent bacterial growth in the filters (see chapter 8). It is recommended to use an enclosed container for this purpose, with a clampable filling tube to connect to the CytoSense and a clampable vent to prevent the release of preservative fumes.
    2. Depending on the colour of your local (sea) water it is advised to use destilled water as a sheath fluid since ground and surface water types including tap-, bottle- and even demi-water may be rather rich in humic acid "gelbstoff" which gives a yellowish/orange fluorescence that increases background noise (reduces sensitivity for the smallest picoplankton).
2. Power down the CytoSense, make sure the Sheath Pump is **NOT** running.
3. Disconnect the circulation bypass and connect for external sheath operation (see figure 8.2). Make sure that a tube which directs sheath fluid into the CytoSense is reaching the bottom of a beaker with fresh sheath fluid. A tube with sheath fluid going out of the CytoSense should be in an empty beaker.
4. Close the waste outlet line to prevent siphoning.
5. To turn on the sheath pump, open *Instrument Control* window in CytoUSB and click a tick next to *Sheath Pump* (it will become green).
6. When most of the fresh sheath fluid is pumped through, stop the Sheath Pump by clicking again the tick from the previous point.
7. Restore operational arrangement of the sheath tubing.
8. Unplug the waste outlet.

16. SHEATH FLUID UNIT: REPLACING THE FILTERS

This CytoSense handles biological material. It is normal practice to add poisonous preservatives to the working fluid to prevent bacterial growth in the filters, but high bacterial concentrations are not impossible.

WARNING: The fluids and the filters must be treated as dangerous contaminated material and should be disposed accordingly.

1. Power off the CytoSense and take it out of its housing.
    1. Disconnect the power cable from the instrument (top side).
    2. Disconnect the USB cable from the machine (top side).
    3. Remove the fuse from the instrument (located in front of the battery; open the fuse holder and remove the fuse).
2. Take off the vent filter assembly.
3. The capsule filters are secured in place by two plastic strips. Unscrew these strips and take out the filters. Take the remaining tubing off the filters.
4. Place the used fluid in a sealed container and follow manufacturers safety guidelines for safe disposal of preservative containing fluid.
5. Slide the tubing on the new filters and put the filters in place and fasten the plastic holder strips again.
6. Mount the new vent filter assembly on top of the top filter and connect the tubing as in figure 8.2

Filling the filters

The new filter(s) contain air which will have to be replaced with sheath fluid:

1. Make sure the CytoSense is unpowered. The sheath pump should be off.
2.
3. Take the syringe connector off and close the filter with a luer cap.
4. Restore the sheath tubing connection with the optical module as in figure 8.2. Make sure everything is tight, clean any salt water spills and dry with some cloth. Check if any clamps are removed.
5. Power the unit and let the sheath pump run for a few minutes to remove air bubbles from the tubing. If any air accumulates in the filters, let it out through the vents by pumping fluid in with the sample pump or directly by refilling with the large syringe.

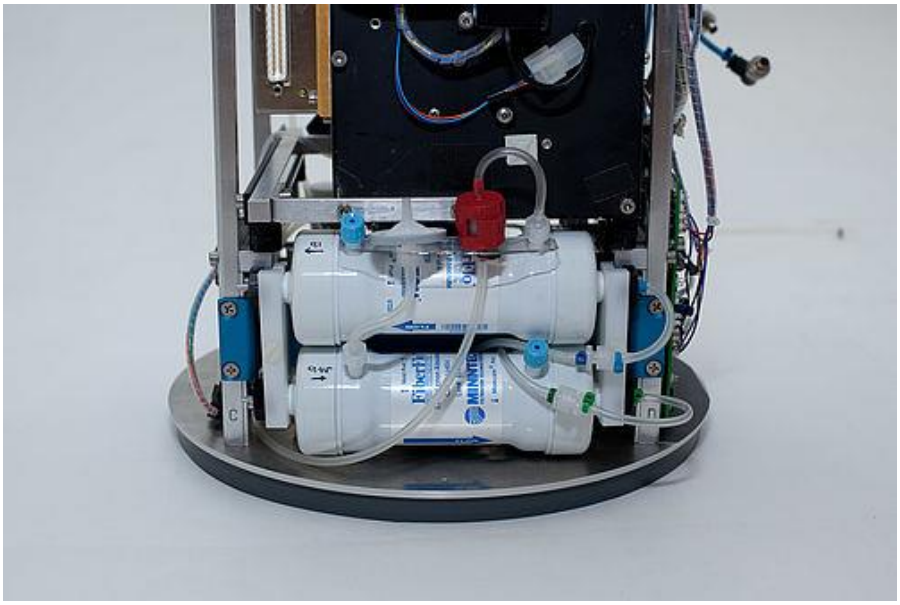


Figure 16.1 Filters

17. INJECTOR: DESIGN AND REMOVING AIR BUBBLES

Design

The injector is specially designed to obtain a stable sample core without the unwanted high fluid accelleration normally encountered in standard flowcytometers. The core remains reasonably stable on moving platforms.

The CytoSense injector has a two-stage injection design (figure 17.1). Sheath water enters the unit below, flowing upward through small peripherial channels and down again in wider channels and finally upwards through a central capillary. This proces is repeated in the second stage, and the central capillary flows into the measuring cuvette.

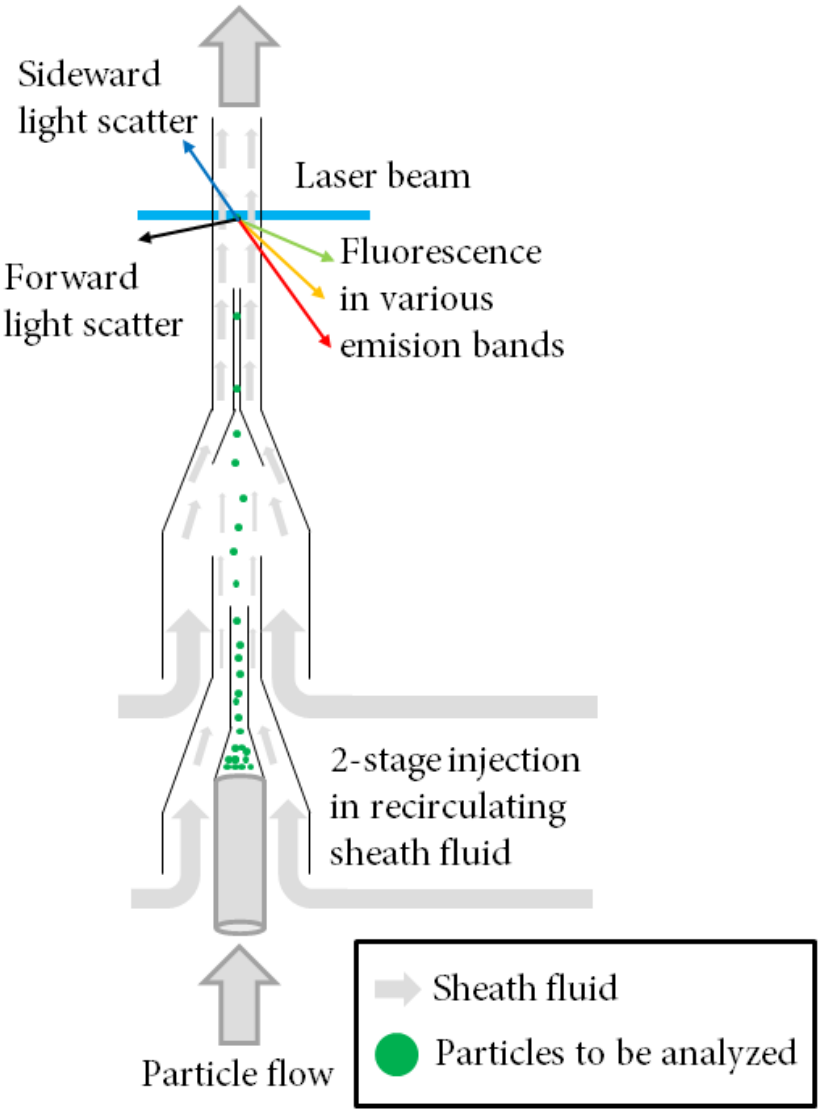


Figure 17.1 Two-stage injection design.

It is advised not to let the system take in any air through the sample line, because air bubbles in the sample fluid are transported through the system, passing the laser beam (generating data), and finally accumulate in the (sheath) filter system.

Although unwanted, the sheath fluid may also contain air bubbles. The injector is designed in such a way that these airbubbles do not enter the central measuring stream but escape from the green zones (figure 17.2) through a by pass line.

In some cases, particularly after servicing, filter replacement etc., an airbubble may get stuck on the central downward flowing pipes (red arrows in figure 17.2). This causes the sample core to be unstable and leads to bad data quality.

Removing air bubbles

To remove an air bubble:

1. Try to squeeze with your fingers the sheath inlet tube for 3-4 seconds (while the sheath pump is activated). This causes a pressure buildup which may release the airbubble when you release your fingers. If that does not help see the next step.
2. Pump a detergent solution or alcohol in with the sample pump. Wait until the sample line is purged and the solution has reached the injector.
3. Stop the sheath pump for ca. 1 minute while keeping the sample pump running. The soap or alcohol will disperse through the lower part of the injector. Wait a few minutes.
4. Switch the sheath pump on again and check if the bubbles are gone. After this procedure let the sheath flow circulate for half an hour at least to clear all dispersed particles from the injector.

Another approach to remove air bubbles: shut down the CytoSense power

1. Clamp off the sheath supply line between the blue luer connector and where it comes out of the filter.
2. Open the blue luer connector and attach a full syringe on the side that leads to the injector and pull/push the fluid in and out with some force until the bubble(s) are gone.
3. Connect the supply line again.
4. REMOVE THE CLAMP.
5. Power up CytoSense again and let the sheat fluid run for some minutes.

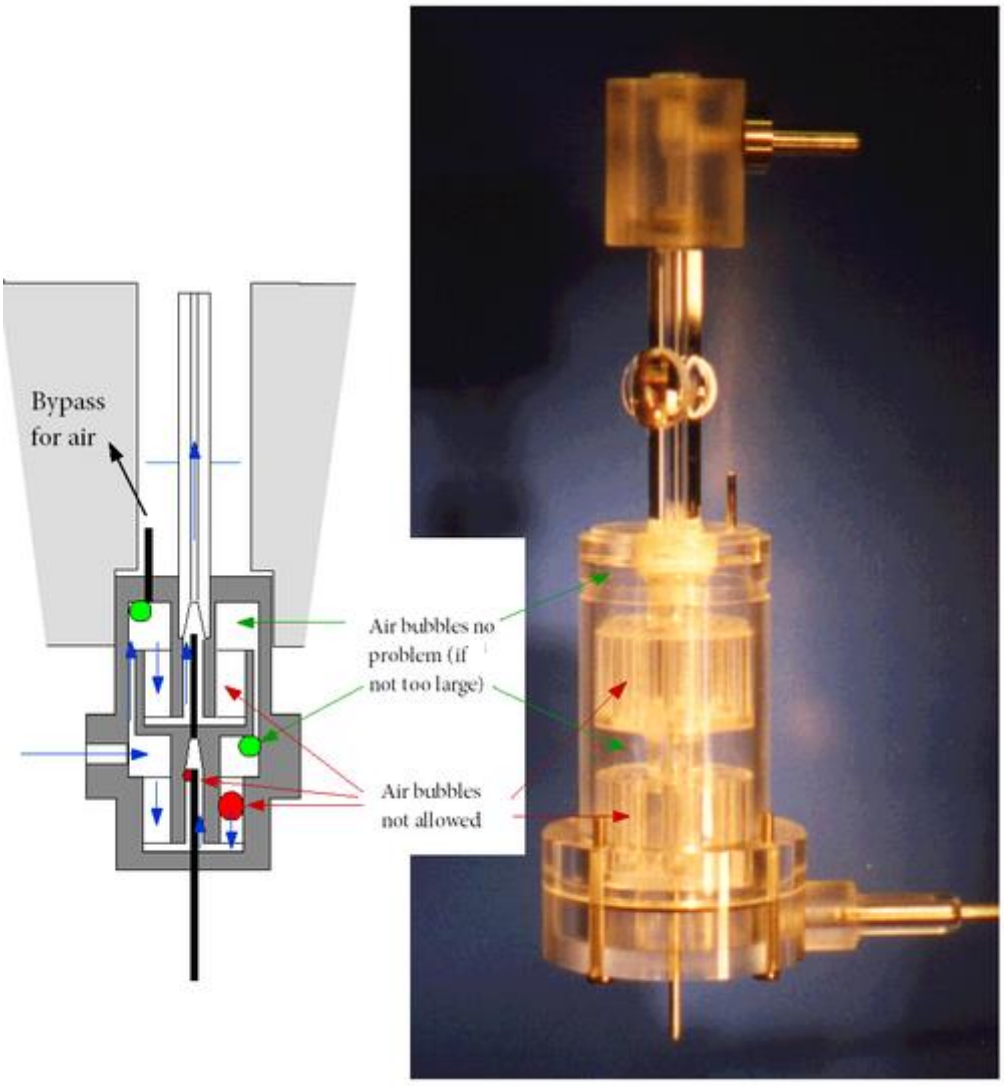


Figure 17.2 Air bubbles in the injector

18. FLOW CUVETTE: INSPECTION AND CLEANING

The optical unit contains a CLASS 3B laser emitting visible light. The laser is fully integrated into the instrument for operator safety and the beam will not be exposed, even when taking the instrument out of the hull. However, take all necessary precautions before opening the optical unit inside this CytoSense.
WARNING: Never look into the laser beam as this can cause serious eye damage and possible blindness.

This CytoSense handles biological material. It is normal practice to add poisonous preservatives to the working fluid to prevent bacterial growth in the filters, but high bacterial concentrations are not impossible.
WARNING: The fluids and the filters must be treated as dangerous contaminated material and should be disposed accordingly.

Inspecting the Cuvette

To inspect the cuvette exterior:

1. Make sure the CytoSense is unpowered
2. Take the red plastic left and right front covers off as in figure 18.1.
3. Take the black middle cover off as in figure 18.1.
4. The cuvette is now visible from the sides.
5. Place the viewer in the hole of the flange.
6. Use the viewer to inspect the position of the laser beam and the sample core and possible contamination (scattering) of the cuvette walls. The complete black aluminum front plate can also be removed to obtain more access in case the outside of the cuvette needs cleaning.

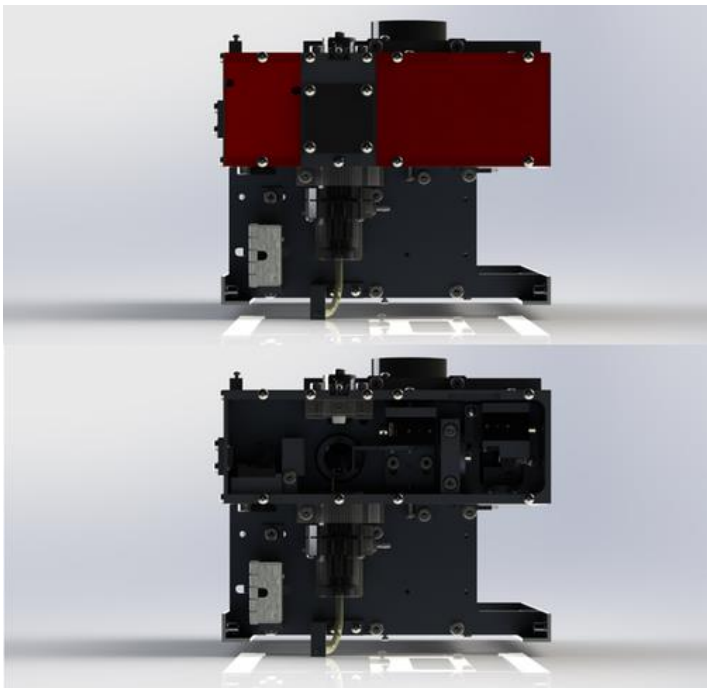


Figure 18.1 Cuvette Inspection

Cleaning the Cuvette

Dirty cuvette walls cause background scattering, particularly on the forward scatter signal, which may show up as an increased noise level.

Flushing the cuvette and the injector with detergent can be tried, but brushing works best in our experience. Special tool: small brush (for example ultrasmall interdental microbrush that fits into a 1mm^2 cappilary). These brushes can be purchased at many drugstores or ordered from CytoBuoy b.v.

1. If you need to slide out the optical module to get access, first switch down power! Clamp off both sides of the sheath fluid tubing near the injector to prevent any spillage of water out of the top. Water that runs down the cuvette on the outside will cause deposit which will need to be wiped off. It is better to prevent this, so keep a paper tissue ready to use. Unscrew the plug screw on top of the fluid outlet.
2. Insert the brush into the cuvette and clean by gentle movements. The brush should be inserted minimally 45 mm downward because the laser intersects the cuvette 40 mm below the top of the outlet.
3. After brushing, gently screw the small plug screw in its place. Check first if the O-ring is in its place. Do not apply to much force as this may damage the thread in the perspex outlet. The plug will seal on the O-ring.
4. Wipe off any fluid.
5. Remove any clamps before running the sheath fluid again.

19. ALIGNMENT OF THE SAMPLE CORE

The optical unit contains a CLASS 3B laser emitting visible light. The laser is fully integrated into the instrument for operator safety and the beam will not be exposed, even when taking the instrument out of the hull. However, take all necessary precautions before opening the optical unit inside this CytoSense.
WARNING: Never look into the laser beam as this can cause serious eye damage and possible blindness.

For the system to work, the sample core needs to be aligned in the center of the cuvette. This can be accomplished by adjusting the screws in the injector (figure 19.1).

Before the sample core is aligned, verify that there are no airbubles or other problems that can cause the sample core to be misaligned.

1. Check the system for air bubbles, if any are present follow instructions in chapter 17.
2. Power down the machine and remove the power and USB cable.
3. Make sure that the instrument is not in its hull.
4. Remove the fuse (figure 4.1).
5. I present, remove the Imaging-in-flow module; else remove the front plate to access the flow cell.
6. Remove the 4 screws that hold the optical module in place.
7. Remove the airduct at the back of the machine and gently slide the optical module forward.
8. Mount the viewer on the optical module and fasten with 2 screws.
9. To make the sample core visible, connect the sample tubing to a source of alcohol (70%).
10. Turn on the sample pump (inwards) at high speed for a couple of seconds to draw in the alcohol.
11. To align the sample core, adjust the three screws that hold the injector in place. By adjusting the three screws, the bottom needle of the injector will move the sample core.
12. Inspect the core in with the viewer, if the core is not clealry visible, add some beads to the alcohol, this will provide a lot of scatter which is clearly visibile.
13. Make sure the core is aligned in the center between the two flow cell walls
14. To align the sample core, it needs to be aligned from 2 axes. To inspect the alignment of the 2nd axis, please follow the remaining steps.
15. Remove the beamstopper plate on the side of the optical module, this allows the laser light to come out of the machine.
16. Using a sheet of paper, localize laser light that comes out of the machine.
17. The sample core will be visibile on the paper as a dark vertical line (second axis).
18. The flow cell walls are also visible as vertical lines (you might need to move the laser spot to see them (figure 19.2). By adjusting this screw, the laser spot will move from left to right on the projection on the paper.
19. Mark the center of the flow cell on the piece of paper.
20. Align the laser spot on the center of the flow cell.
21. Keep adjusting the screws of the injector untill the sample core is aligned in the center on both axes.

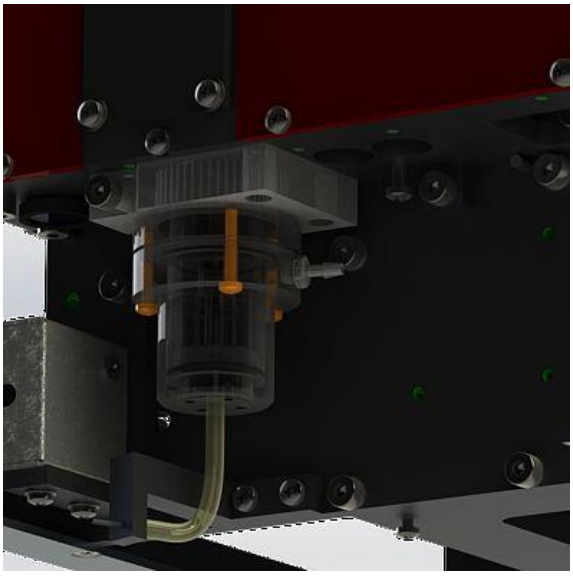


Figure 19.1 Injector with highlighted screws in yellow

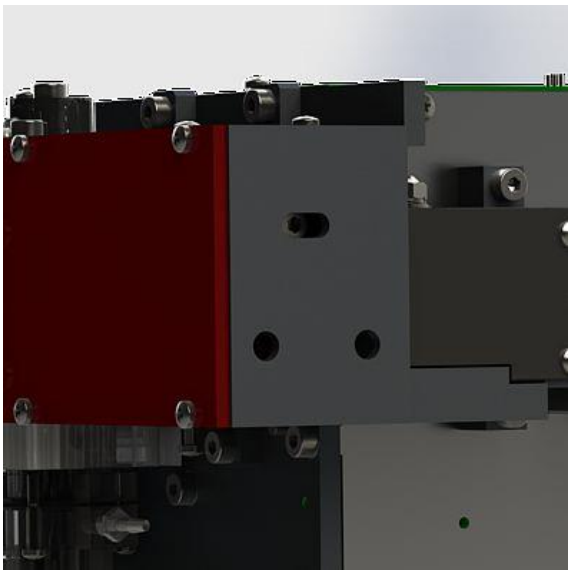


Figure 19.2 Adjusting the position of the laser spot

20. SAMPLE PREPARATION AND FIXATION

Sample preparation

Gymnodinium corii. Size and chlorophyll autofluorescence the cultures was preserved very well for up to four months. This was however only a single species and is not representative of heterogeneous phytoplankton communities.

Recently a simple protocol has been developed which allows picoplankton sample concentration for flow cytometry. This protocol can be used for the least represented group of picoeukaryotes (down to 102 cell per ml) avoiding any cell lost or cell damage (Biegala et al. 2003). The main improvement was the use of a cell surfactant (Pluronic F-68) combined with centrifugation. Generally, the ideal situation is to analyse fresh samples soon after collection without pre-concentration.

Biegala, I. C., F. Not, D. Vaulot, and N. Simon. (2003). Appl. Environ. Microbiol. 69:5519-5529

Hofstraat J.W, W.J.M. van Zeijl, J.C.H. Peeters, L. Peperzak and G.B.J. Dubelaar. (1990) In: H..O. Nielsen (Ed), Environment and Pollution Measurements Sensors and Systems, S.P.I..E. Proceedings 1269, pp. 116-133, Int.Soc.Opt.Eng, Bellingham, WA.

Premazzi G., F. Bertona, S. Binda, G. Bowe and E. Rodari. (1992). EUR 14806, European Communities - JRC.

Vaulot D., Courties C. and F. Partensky: (1989) Cytometry 10(5):629-636.

21. CONCENTRATION: CALIBRATION AND ERRORS

The concentration of detected cells is basically determined by the counted number of particles, the time interval of this count and the sample flow rate.

Calibration of the flow rate is a relatively simple exercise: fill a calibrated syringe or glass pipette with water, connect it to the sample input tube and let the sample pump run constantly (the sheath pump should also run). Check the time needed to pump a few millilitres into the instrument with a stop watch. Check this for a few different sample pump speeds. The actual pump speed can be entered in the calibration utility of the CytoUSB program (cf. chapter 11, point 15). Checking the counting performance of the instrument should be done with a suspension of calibration beads of known concentration (chapter 11chapter 12

22. SHEATH FILTERS: BACK FLUSHING

This CytoSense handles biological material. It is normal practice to add poisonous preservatives to the working fluid to prevent bacterial growth in the filters, but high bacterial concentrations are not impossible.

WARNING: The fluids and the filters must be treated as dangerous contaminated material and should be disposed accordingly.

BACK FLUSHING THE SHEATH FILTERS

In this case the system and filters are flushed back with clean particle free water and the old sheath fluid will be expelled. Please take a moment to observe the fluid path for back flushing from the schematic layout shown in Fig. 63, before you proceed with the following steps:

1. Prepare at least 4 liters of clean particle free fresh water.
2. Power off the CytoSense and take it out of its housing.
    1. Disconnect the power cable from the instrument (top side).
    2. Disconnect the USB cable from the machine (top side).
    3. Remove the fuse from the instrument (located in front of the battery; open the fuse holder and remove the fuse).
3. See figure 64.
    1. Disconnect the luer connector in the cuvette return tubing and insert a filling and a waste outlet tube according to the figure.
       Note: this is different from the setup used for changing sheath fluid/using external sheath fluid!!
       Do not forget to plug off the waste outlet line from the vent filters to prevent siphoning.
    2. See figure 65. Disconnect the luer connectors in the suction and pressure tubing of the sheath pump and connect them again cross wise: the green to the blue and vice versa, as shown in the figure.
4.
5. When most of the fresh fluid is pumped through: stop the pump. Restore the original situations A (as in Fig. 23) and B (as in Fig. 41) and do not forget to:
    1. Unplug the waste outlet.
    2. Add some biocide to prevent bacterial growth in the filters.
6. Place the expelled fluid in a sealed container and follow manufacturers safety guidelines for safe disposal of preservative containing fluid.