Procedure summary

This procedure outlines the proper operation and maintenance of the Fluid Imaging Technologies FlowCAM.

1.1. Related Procedures

Mini-Beadbeater-24	LC-06-001-011

1.2. Procedure impacts and concerns

Safety	Proper PPE for this procedure: safety glasses and gloves. Nitrile gloves should be worn when handling pond samples.
Quality	Ensure that samples are handled in accordance with sample handling and on site transport procedure.
Delivery	NA
Environmental	NA
Cost	NA
Compliance	Compliance with Sapphire Energy, Inc. Chemical Hygiene Plan and IPM policy is required. See 29 CFR 1910.120 and 1200. An authorized user list, MSDS's and label information will be available for easy reference in a binder in the administration building.

1.3. Responsibilities and owners

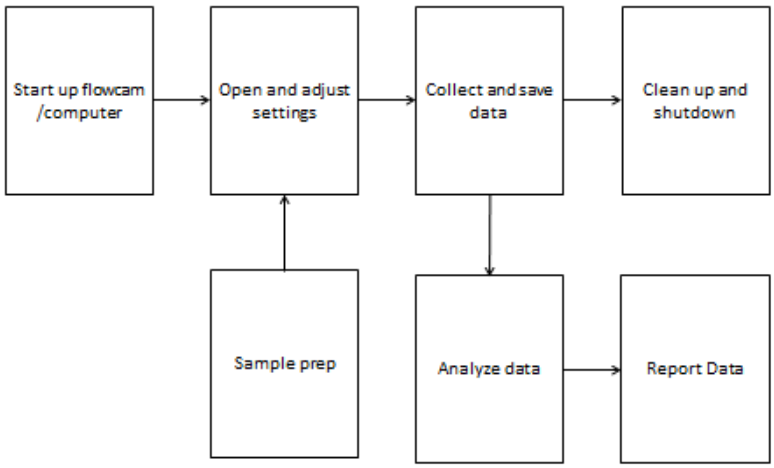
Document Owner	Manage content and distribution	Phil Lee
Process Owner	Responsible for content and process validation	Phil Lee
Plant Manager	Responsible for implementation and conformance	Becky Ryan

2. Process

2.1. Process description

The FlowCAM allows for high throughput analysis of particles in a moving field. This document describes proper sample preparation and analysis using the FlowCAM. An in-depth manual is available for the FlowCAM, and should be referenced when possible for details of procedures not within the scope of this SOP.

2.2. Process diagram:



2.3. Process steps

1) Obtain materials required for procedure:

Per sample:

- 5ml "preview" tube (fisher snap cap culture tube) to collect sample after filtering
- Screw cap microtube (if bead beating is required)
- 0.7mm Zirconium beads (if bead beating is required)
- 1.5-2.0ml Eppendorf tube or equivalent for diluting sample prior to loading
- Filter unit appropriate for flowcell in use (for 50µm flowcell = 50µm filter, e.g. Partec Celltrics 50µm disposable filters available in lab) for pre filtering sample prior to loading on FlowCAM to prevent blockage of the flow cell
- A FlowCAM
- Media for sample dilution
- Sterile water
- 4-6 % v/v bleach solution
- Pipettes and tips
- Samples

2) Prepare Culture Sample

a) Label all tubes with each sample name.

b) [Optional] Bead beat sample to break up flocks.

If the sample contains flocks of algae, bead beating must be used to break up these flocks, thus enabling the individual algae to be analyzed. For some samples where flocking is not evident this step may be omitted from the procedure. Refer to SOP LC-06-001-011 for proper use of the beadbeater using the following parameters:

i) Add approximately 0.5 g of 0.7mm Zirconia beads into a 2ml screw cap vial with conical bottom.

ii) Add 1ml of culture sample by pipette and secure tube top

iii) Bead beat the sample for 3 seconds

c) Place filter unit over collection tube

d) Pour sample into filter unit to remove large debris that may clog the flow cell. A gentle tap may be

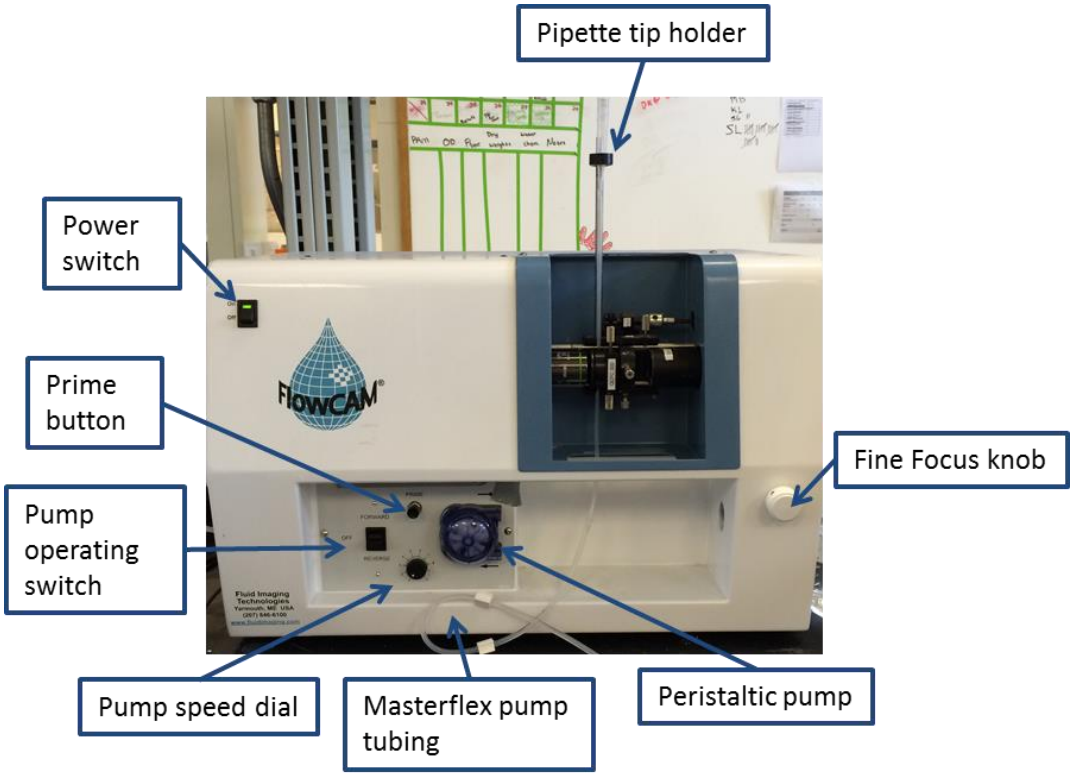
required if the sample does not easily flow through the filter. Recommended filter sizes for different flow cells is shown in the table below.

Flow cell	Recommended filter pore size
FC50	35-50 µm
FC100	100 µm
FC300	300 µm
FC600	600 µm

e) Dilute filtered sample in appropriate media or buffer into labeled Eppendorf tube or other appropriate vessel.

Dense cultures should be diluted to obtain a final particle concentration that does not yield multiple particles per image in FlowCAM data. With current typical pond cultures, a ten-fold dilution is sufficient. This dilution will be dependent on the cell size/morphology, carrying capacity, typical culture density etc.

f) Record the dilution factor for future particle calculations.



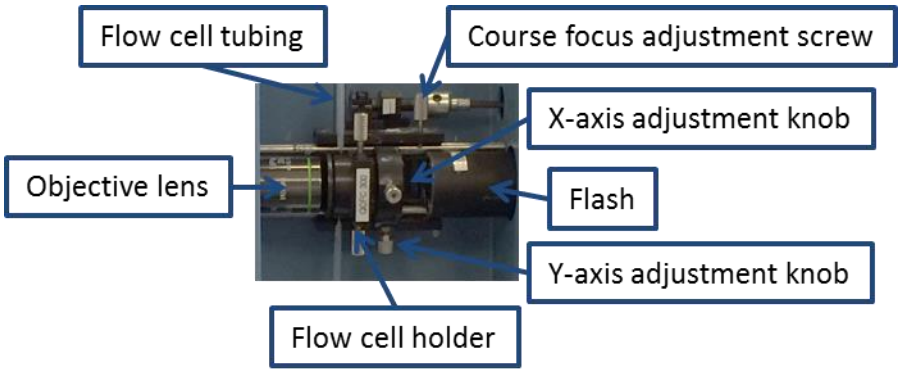


Figure 1. Anatomy of a FlowCAM

3) Setup the FlowCAM Hardware

a) Based on particle size of interest, determine the proper flow cell size to use. 50 µm, 100 µm, 300 µm and 600 µm flow cells are available. 50 µm is used for general analysis at IABR and LCTS sites and should already be installed. If a different Flow Cell than installed is required then see "Replacing Flow Cell" procedure in the FlowCAM manual for instructions on installing or switching out Flow Cells (p23-24).

b) Turn on the FlowCAM (if not already powered on). Power buttons are located at the front (top left) and rear of the machine (top left when facing the front of the machine). The FlowCAM has an integrated computer which will start up Windows.

c) Install pump tubing:

i) Remove the blue case on the front of the pump head by pulling on the plastic area between the inlet and outlet (see picture below)

ii) Remove the white roller assembly

iii) Wrap the tube around the roller assembly (paying attention to the flow direction) and slide back into the pump head making sure the tube is seated between the rollers and the edge of the pump head enclosure, and not sitting on top of the rollers. While holding the ends of the tubes by the spacers, snap the blue enclosure back onto the pump head. ***It is important to note that tubes should not be left in the pump head with the pump turned off for long periods of time such as overnight or weekends.***

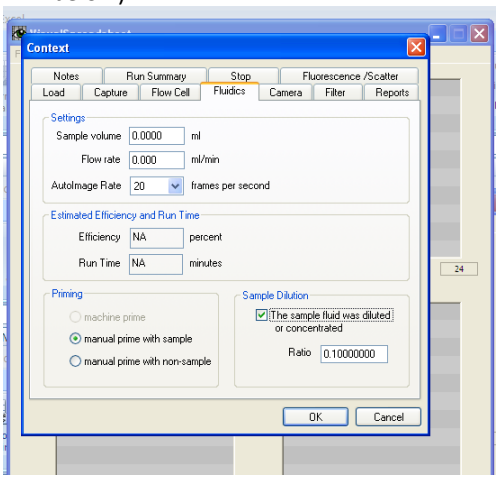
4) Setup the FlowCAM Software

a) Start the FlowCAM software by opening VisualSpreadsheet. An icon for VisualSpreadsheet can be found on the desktop. Upon opening the software you will be prompted to choose the appropriate objective. The 20x objective is used for standard sample analysis at LCTS and IABR sites when using the 50 µm FlowCell.

b) Adjust data collection (Context) settings. Current Context settings will be as used by the current logged on user from the previous run or set as factory default the first time a user has logged on. Preset Context settings can be loaded from saved files or manually adjusted as necessary for the

current experiment.

i) If adjustments are required: Click Setup→Context to bring up the Context menu (see picture below)



ii) To load a context settings file: Select the Load tab, click "Load a context file…" and locate the context file appropriate for the current sample.

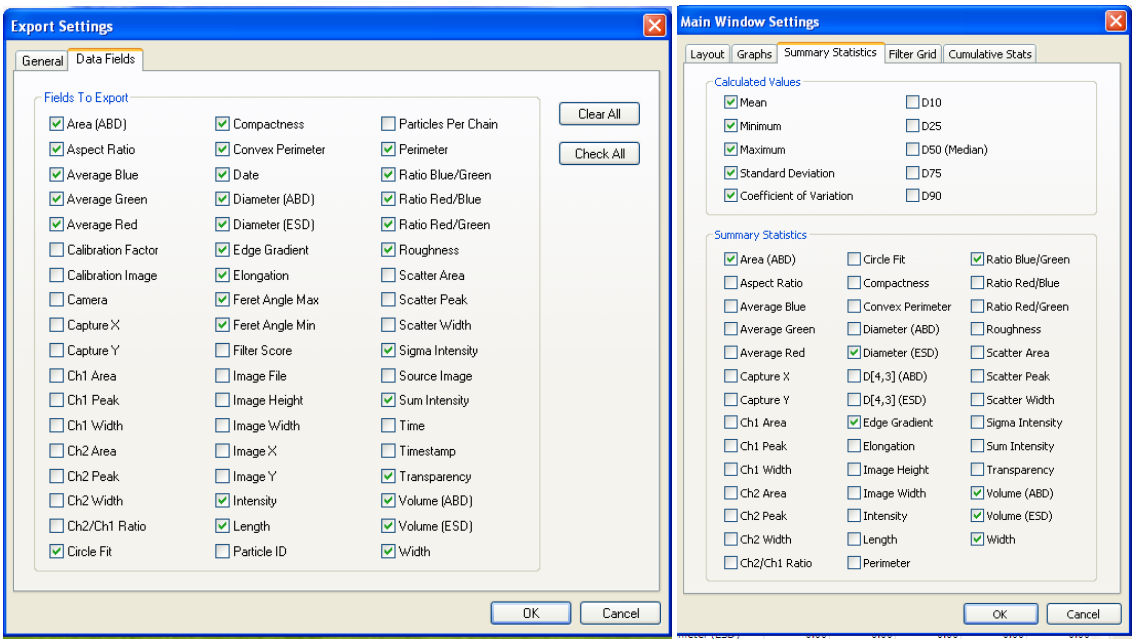
iii) To manually set context settings, navigate through the tabs within the context menu and adjust settings as necessary. A list of frequently adjusted settings can be found in the Appendix.

iv) After adjusting context settings they can be saved locally or elsewhere using the "Load" tab within the context settings menu (Setup→Context).

c) Adjust data export settings. Export settings set which data points are exported in excel format in data or data summary files.

i) Click Preferences→Export Settings and check which datapoints are desired to exported data. Suggested example shown below.

ii) Click Preferences→Main window settings and check which summary data are desired. Suggested example shown below.



5) Rinse and prime the Flow Cell and pump tubing

a) Place pipette tip into pipette tip holder and attach flow cell tubing

b) Load 1 mL of purified water into the pipette tip located in the pipette tip holder. Any solutions used during flowcam should be free from debris that could block the flow cell. ***It is advisable to use pure sterile water and not use spray or squirt bottles for this purpose.***

c) Move the peristaltic pump switch to forward position and press the prime button. Allow the full volume of water to rinse the flow cell and fill tubing from sample loading tip to the pump (additional water may need to be added for this and to avoid air entering the system).

d) Once all water has filled the system and level is at the bottom of the sample funnel / tip, press the prime button and move the peristaltic pump switch to the off position to stop the flow.

e) Check for bubbles and remove any if necessary (p28 FlowCAM manual)

6) Load the Culture Sample

a) Pipette 500 µl of the diluted culture sample and load it into the sample holder of FlowCAM.

b) Move the peristaltic pump switch to forward position with the pump speed on it lowest setting.

c) Select Setup→Auto image (no save) mode (F7 shortcut key)

d) Check sample is in focus and adjust fine focus adjustment knob as needed to bring cells into focus (p32 of FlowCAM manual). It is easiest to adjust focus when the pump is off.

e) Check intensity mean and max are within limits. Intensity mean should be 180-200 and the max <255. If values are outside these limits adjust Gain and Flash duration under camera settings (see p31 FLowCAM manual)

f) Check that no blockages are present and sample images appear to representative of the sample being analyzed.

g) If multiple particles are present per image, dilute the sample further until only single particles are present in each image. Record the final level of dilution.

h) When sample images and parameters are within desired ranges, close no save mode and proceed with data collection.

7) Initiate sample run.

a) Select Analyze→AutoImage mode from the software menu to begin image capture.
b) Adjust data collection information as appropriate (e.g. number of images to collect etc.)
c) Select the folder on the computer where the file is to be saved. Data manipulation and analysis is faster when files are located on the hard drive of the FLowCAM computer but all work should be saved onto the networked drives as soon as possible after analysis is complete.
d) Allow the hardware to run until the stop function is triggered and the software completes image capture. Add additional sample if needed during this process to prevent air entering the system.
e) When data collection has completed allow the sample to empty from the sample funnel/tip.
f) Add 0.5-1 ml sterile water to sample funnel/tip, with the pump in forward direction press the prime button to rinse the sample funnel/tip and flow cell between samples in order to remove any cells from the previous sample.
g) Repeat above steps from **5. a)** for further samples until all sample collection is complete.

8) Stop the Hardware

a) Move the peristaltic pump into the off position.

9) Rinse the Flow Cell

After all samples have been run for the day the tubing and flow cell should be cleaned and left empty.

a) Load 1 mL of a 4-6% (v/v) bleach solution into the FlowCam sample holder.
b) Move the peristaltic pump to the forward position and press the prime switch. Allow the full volume to pass through the flow cell.
c) Load 1ml of purified water to the FlowCam sample holder and allow the full volume to pass through the flow cell and all tubing.
d) Remove tubing from the peristaltic pump
e) Discard sample tip (or remove funnel for cleaning)
f) Place flow cell input tubing in a downward configuration to prevent dust/debris from entering during storage
g) Shutdown the computer/flowCAM

10) Analyze FlowCAM data using Classification

Data can be exported into excel format at any point after collection by opening a list file and selecting File→Export Data summary / Export data. The steps below include ways to separate out particles of interest prior to exporting data. The "data summary" file contains data on the population of particles whereas the "data" file will contain data on each individual particle image.

The classification window allows a user to separate images within a list file into different classes, either manually or automatically by using predetermined filters. All the data corresponding to the images within classes can then be exported or analyzed together. Further information about classification functions can be found on p113 of the FlowCAM manual.

a) Open a list file by choosing File→Open List and choosing required sample list file
b) Press F3 followed by F2 to show all images in the list file.
c) Open the classification window from the list file overview by choosing File→Open Classification window
d) In the Classification window chose File→Open classification
e) If a template exists locate it after clicking on the "find Template" button
f) Name the file in the Name box and click on the "create" button. File naming convention is

"YYDDMM XXXX" where XXXX represents the pond name. In order to utilize downstream data manipulation macros in excel the files must be saved using this nomenclature.

g) If you are using a previously saved template tabs will appear representing each class within the classification. If this is a new classification then classes can be added by clicking Classification→ New Class. Classes can be edited by choosing Class→Edit class. In this menu filters can be added to a class to enable auto classification.

h) If filters are loaded into the different classes, initiate auto classification by clicking Operation→Run Auto classification. Images should be placed into the different classes automatically. Any not meeting the parameters of the filters used will be displayed in the original list file.

i) If no filters are used or to move images between classes or from the list file into classes, choose images by using the left mouse click. When all images are chosen, right click, chose "move to class", select destination class for selected images.

j) To speed up selection of images they can be sorted in either the classification or list file views by any of the measured parameters. To do this click Sort→ and chose the parameter to sort by. In the list view images can also be sorted by the use of filters.

k) All the images on a screen can be selected by pressing Ctrl+F3 or by holding down the Ctrl key while click and dragging with the mouse (left button). All images in a list file can be selected by pressing Ctrl+F2

l) Once all the images have been classified the summary or detailed data can be exported into Excel format by clicking File→Export Data summary / Export data

Appendix

The table below list common parameters within the context menu (From Setup→Context settings) that may be changed for different types of data collection or flow cell set up. Details on paramters not listed here can be found in the FlowCAM manual.

Menu	Parameter	Typical Settings	Notes
Filter	Basic size acquisition filter	2.0-10,000 microns	Alter to limit the particle size that images are captured for.
Stop	Stop when particle count reaches:	1000-3000	Adjust so that the desired number of useful images is captured. This will be dependent on end use and on how clean the sample is.
Camera	Gain	100	Used to bring image intensity into range
Camera	Flash duration	33	Used to bring image intensity into range
Fluidics	Sample dilution	0.1	Use this for a 10X dilution which is typical for routine FlowCAM on pond samples
Capture	Acceptable region	Left 1, Right 1278, Top 1, Bottom 958	This creates a 1 pixel boundary around the field of view so that partial images (that cross the boundary) will not be saved in the final datset

Document control

Revision history

R0 – Philip Lee	11/19/2014

Document approval

<Name> Xx/xx/20xx

Document reviewers

Phil Lee 11/17/2014

Risk analysis

<Risk name>	<Mitigation plan>	<Owner>	<RPN>