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RE	Restricted to a group specified by the consortium (including the Commission Services)	
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1. Key steps

Isolating micro-algae from natural samples involves four major steps. For each of these steps there are many variations possible (e.g. are samples to be concentrated or not, type of medium used) and one should test as many combinations as possible, since each variation will be probably lead to different type of micro-algae being isolated. In some cases, isolation by flow cytometry is unsuccessful and we advise to always use alternative methods at the same time (e.g. serial dilution, pipette isolation).

1. Sample collection and enrichment
2. Flow cytometry sorting
3. Culture screening
4. Culture characterization and purification

2. Material

2.1 Equipment

- Sorting flow cytometer: FACSAria (Becton Dickinson)
- Analytical flow cytometer C6 Accuri (Becton Dickinson)
- Laminar flow cabinet
- Inverted microscope equipped with epifluorescence Olympus IX71
- Incubation chamber with light:dark cycle and blue filters
- Tangential flow cartridge (Vivaflow 200) with peristaltic pump

2.2 Strains, solutions and chemicals

- Culture media: K (for eukaryotes) and PCR-S11 (for cyanobacteria) -see recipes in Appendixes.
- Pluronic F-68 solution (Sigma, P5556)
- Antibiotic PNS (Penicillin-Neomycin -Streptomycin; Sigma, P4083)

2.3 Culture ware

- Multiwell plates: 24, 48, 96 well
- Polystyrene culture flasks with or without ventilated caps: 50 mL (Sarstedt ref 831810.002 or Nunc ref 136196)
- Glass tubes: 5 mL, 15 mL
- Polystyrene tubes (CML ref TCU12PS25): 10 mL

3. Sample processing

3.1 Sample collection

Samples are collected

- during cruises from CTD bottles
- from sediments using a corer
- from sediments by SCUBA diving
- from the surface of marine invertebrates (sponges, corals) by SCUBA diving

3.2 Sample concentration and filtration

Samples can be used:

- un-concentrated
- pre-filtered through 3, 1 or even 0.6 µm. We recommend to use gravity filtration (no depression) and using several Nuclepore filters stacked together
- concentrated by tangential flow filtration (TFF). We recommend Vivaflow 200, 0.2 µm, cartridge, which allows to concentrate a few liters to 20 mL in less than 30 min. Usually recovery is very good, in excess of 50% and fragile cells (e.g. flagellates) resist very well this treatment.

3.3 Sample incubation

It is best to perform the flow cytometry isolation immediately after sample collection on board the ship, but in many cases this is not possible. So samples must be maintained on board until brought back to the laboratory. Under such cases it is best to enrich the samples, because if not it is very likely that nothing will be left upon arrival (except for the case of TFF concentration, see below).

Until processed, samples should be maintained in culture cabinet at the temperature and light level to which there were collected. Usually we maintain surface samples at 100 µE and DCM samples at 10 µE blue light in 12:12 light:dark cycle. For tropical cruises we use temperatures in the range 20-22°C, for temperate in the range 13-15°C and for polar waters 4°C. Samples are best maintained in 50 mL cultures flasks.

Samples can be enriched with different dilution of the recommended media (K or PCR S11). If one wants to eliminate diatoms, add a few drops of Germanium oxide. One can use full strength medium (e.g. 0.1 mL of sample into 20 mL of medium), medium/5 (e.g. 4 mL of sample for 1 mL of medium) or medium/100 (e.g. 0.2 mL of medium for 20 mL of sample). Samples should be checked if possible by inverted microscopy to monitor growth.

We noticed that samples concentrated by TFF could be kept without any media enrichment for quite a long time, as if a natural food web maintained itself.

It is very critical to minimize the time of transfer between the boat and the laboratory. If time of transit is beyond one day, try to expose the flasks to natural light conditions but never ever to direct sunlight (use a North facing window). The "Cyanobacteria survival box" developed by MACUMBA partner 5 (Cyanobiotech) could be used for maintaining samples under optimal conditions.

4. Flow cytometry sorting

The protocol is for the BD FACSAria equipped with 488 and 633 nm lasers and standard filter setup (Figure 1) but other instruments such as the BD Influx can be used as well.

4.1 Preparation of flow cytometer

Figure 1 : BD FACSaria flow cytometer

- In order to prevent sample contamination, the flow cytometer is cleaned the day before sorting, with a succession of 3 solutions, bleach (10 mL of 2.6% (vol/vol) bleach solution into 2 L of DI water), detergent (5 mL of cleaning concentrate Bio-Rad 161-0722) into 2 L of hot water, around 70-80°C) and DI water. For each solution, a normal fluidics Start Up is performed, the stream is then activated and the solution is let running, with activation of the sample line back flush, for two hours for bleach and detergent and overnight for DI water.
- Natural seawater is prepared the day before cell sorting. Between 2 to 10 L of seawater are filtered by tangential filtration using a Vivaflow 200 cartridge equipped with a 30,000 MWCO RC membrane (Sartorius Biotechnologie SAS, France). Seawater is then autoclaved in 2L glass bottles for 2 hours at 120°C.
- Just before the flow cytometry sorting, a 2 L glass bottle containing autoclaved seawater is placed on the flow cytometer fluidic cart. A 0.22 µm filter unit (Sterivex) is placed at the head of the sheath line and immerged into the sheath liquid. A normal fluidics Start Up is performed.

4.2 Sample analysis

PMT voltages are set as follow: FSC 250, SSC 350, Green 500, Orange 500, Red (450). The discriminator is set on both the red fluorescence and the SSC with threshold values of 200 and 1000 respectively. The sheath pressure is set at 70 PSI and samples are run with a rate of 40 to 50 µL/min. Gates are chosen according to the populations that are targeted (Figure 2).

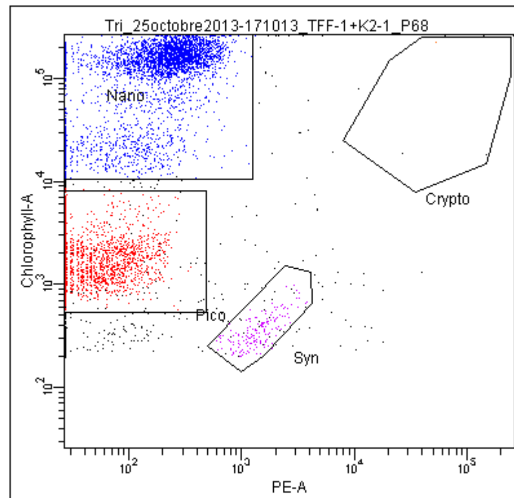

Figure 2: Flow cytometry analysis of a TFF concentrated sample from Roscoff (x=phycoerythrin fluorescence - y=chlorophyll fluorescence). Examples of sorting windows that can be used for isolating microalgae (pico=picoeukaryotes, nano=nano-eukaryotes, Syn=*Synechococcus*, Crypto=Cryptophytes).

4.3 Sorting

For sorting delicate cells the blue 488 nm laser can be reduced with a blue filter to decrease photodamage to the cells. Frequency for sorting is set at 90,000 Hz, Drop 1 positioned at 192 pixels with a Drop Delay of 43.2, and deflection plate voltage at 5,000 V. Cells are sorted in “Purity” mode.
Sorting can be made into 96, 48 or 24 multi-well plates or into glass tubes. In general we do one plate per sample. The plates are prefilled with sterile medium (200 mL for 96 wells, 1 mL for 48 and 24 wells). 48-wells plates seem to provide better results but not always We recommend the following set up to maximize chances of recovery (Figure 3). The first wells receive large numbers of cells (1000, 100, 10, 2) to end up with wells receiving single cells. Single cell isolation is very tricky and success rate can be very low depending on the type of cells (e.g. for picoeukaryotes or cyanobacteria).

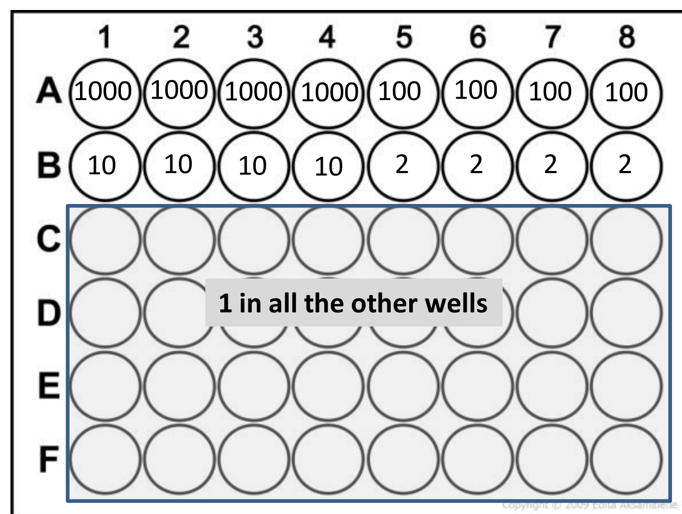

Figure 3. Typical set up for plate sorting: number of cells sorted in each well.

5. Incubation and screening

After sorting the plates are sealed with parafilm to minimize evaporation. Immediately after sorting the plates or tubes are placed under dark conditions for at least 24 hours. This is necessary to allow

cells from the photo damage that takes place during the brief exposure to laser light. Plates are incubated under standard conditions (12:12 L:D cycle, temperature and light depend on the conditions under which the samples were incubated.
Between 7 and 10 days after sorting, the plates are examined by epifluorescence microscopy on an inverted microscope (Figure 4). It is the quickest way to screen whether photosynthetic cells have begun to grow. Positive wells are then transferred first to 24 well plates with 2 mL of medium, which are then transferred after a few days to 50 mL culture flasks. Wells with the fewer number of sorted cells are privileged and more than one well (2-5) should be transferred to culture flasks because such cultures are still sensitive.

Figure 4: Olympus IX71 microscope used for screening plates

After growth in flask has started, cultures are further checked by flow cytometry (e.g. with the C6) and microscopy. If the resulting cultures are still mixed, then a second round of sorting can be performed.

6. Strains that have been isolated by flow cytometry sorting

The Roscoff Culture Collection (http://www.roscoff-culture-collection.org/) contains a large number of cultures that have been isolated by flow cytometry sorting (Table 1). The group that has been most easily isolated with this approach has been diatoms (Bacillariophyceae). However some more delicate groups such as flagellates (e.g. Cryptophyceae) have also been isolated this way.

Table 1: Number of strains of the Roscoff Culture Collection that have been isolated by flow cytometry sorting

Class	RCC #
Bacillariophyceae	66
Prymnesiophyceae	47
Pelagophyceae	46
Prasinophyceae	44
Mamiellophyceae	42
Cyanophyceae	38
Dictyochophyceae	13
Cryptophyceae	7
Nephroselmidophyceae	5
Trebouxiophyceae	5
Chrysophyceae	4
Dinophyceae	2
Chlorarachniophyceae	1
Ulvophyceae	1
Unknown	16

Total	337

7. Optimisation of sorting conditions

Many of the sorting parameters, sorting conditions, and incubation conditions can be optimized. We list below a few of the tests that have been performed. The following strains were used for this purpose: *Micromonas* (RCC 299), *Isochrysis* (RCC 90), *Scripsiella* (RCC 2543) and *Synechococcus* (RCC 1085).

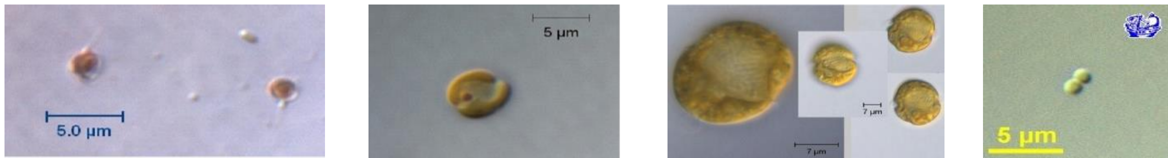

Figure 5: Picture of strains used for testing.
RCC 299-*Micromonas*, RCC 90-*Isochrysis*, RCC 2543-*Scripsiella*, RCC 1085-*Synechococcus*

7.1 Comparison between blue and red laser

One of the key damage experienced by the cell during sorting is photodamage. Therefore we tested whether using a blue (10mW, 488nm) and or a red laser (15mW, 633nm) on two strains (*Scripsiella* and *Isochrysis*) sorted into 96 wells plates with K/10 medium. We sorted either 100, 10, 2 and 1 cells per well. The fraction of wells were positive cultures had regrown from the sorted cells were checked by microscopy 3 weeks after cell sorting (Figure 6). No differences were observed between the use of both lasers: the number of positive wells was decreasing with the number of cells sorted and very similar between blue and red lasers.

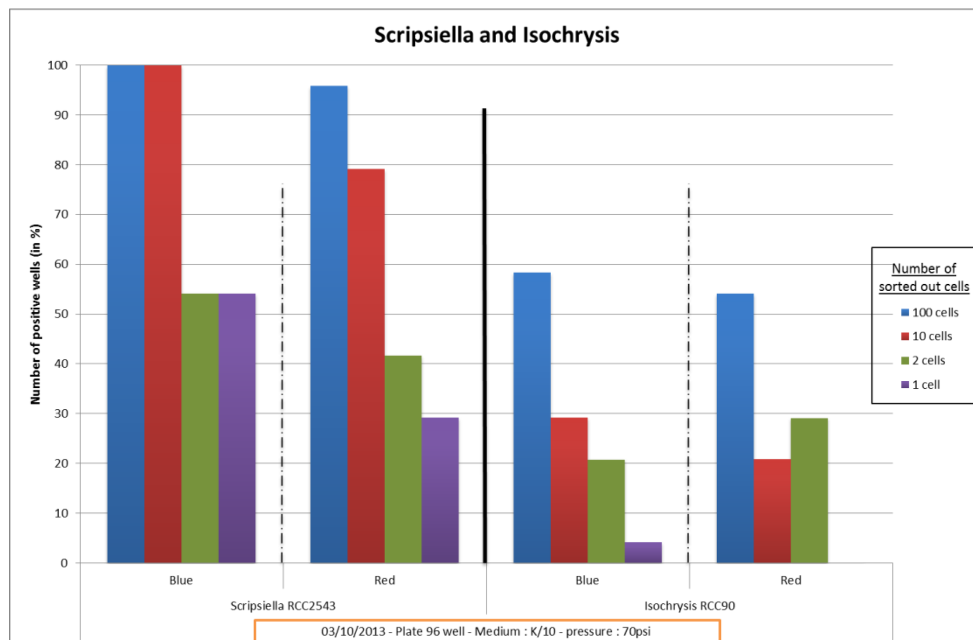

Figure 6: Comparison of sorting recovery for blue vs. red laser for two RCC strains.

7.2 Culture medium concentration

Concentration of the medium into which the cells are sorted (either full, diluted half, 10 times or 100 times) was tested on three strains (*Isochrysis*, *Synechococcus* and *Scripsiella*). We used 48 well plates.

Growth was monitored by flow cytometry during 8 days. Results indicated that concentrated medium is better.

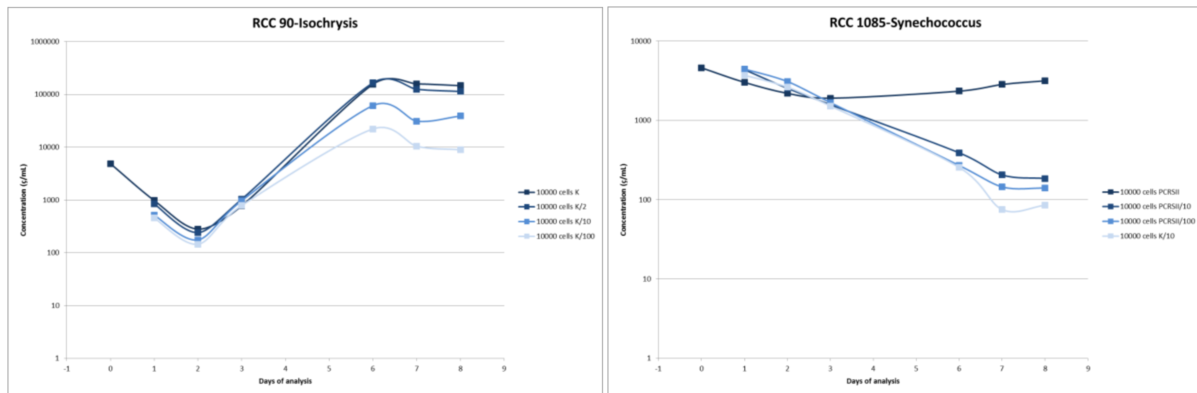

Figure 7: Growth of cultures following sorting of 1000 cells into different concentration of medium

8. References

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Appendix 1: Preparation of K medium

1. Filter 1L of old seawater of at least two months on prefilter and filter 0,2 microns
2. Heat seawater during 20min at 100°C
3. Under hood, t o seawater, add these nutriments beforehand autoclaved (excepted vitamin):

Quantity	*Compound*	*Stock Solution*
1.0 mL	Sodium Nitrate ($NaNO_3$)	75.0 g/L of H_2O
1.0 mL	Ammonium Chloride (NH_4Cl)	2,68 g/L of H_2O
1.0 mL	Na2-Glycérophosphate ($C_3H_7O_6PNa_2$, 5 à 6 H_2O)	3,06 g/L of H_2O
1.0 mL	TRIS-Base (pH7.2)	121, 1g/L of H_2O
1.0 mL	K Trace Metal Solution	(see recipe below)
0.1 mL	F/2 Vitamin Solution	(see recipe below)

4. Filter the medium on 0,2microns

K Trace Metal Solution

To 900 mL of H_2O add :

Quantity	*Compound*	*Stock Solution*
41,6g	EDTA Disodium Salt Dihydrate ($Na_2EDTA.2H_2O$)	
3,15g	Hexahydrated ferric chloride ($FeCl3.6H2O$)	1,5 cc (liquide)
1.0 ml	Sodium Molybdate Dihydrate ($Na_2MoO_4.2H_2O$)	6,3 g/L of H_2O
1.0 ml	Zinc Sulfate Heptahydrate ($ZnSO_4.7H_2O$)	22.0g/L of H_2O
1.0 ml	Cobalt Chloride Hexahydrate ($CoCl_2.6H_2O$)	10.0g/L of H_2O
1.0 ml	Manganese (II) chloride, tetrahydrate ($MnCl_2.4H_2O$)	180.0g/L of H_2O
1.0 ml	Copper(II) sulfate pentahydrate ($Cu SO_4.5H_2O$)	4.9g/L of H_2O
1.0 ml	Selenous acid (H_2SeO_3)	1,29g/L of H_2O

Make final volume up to 1.0L using H_2O. Heat to dissolve

F/2 Vitamin solution

- In 100mL of distilled water, dissolve 0,05g of biotin (vit. H) and 0,05g of cyanocobalamin (vit. B12) = solution A
- In 0,5mL of solution A, add 0,05g of thiamine HCl (vit. B1) and complete final volume to 50mL of distilled water
- Filter sterilize on Millipore 0,22µm
- Store in refrigerator or freezer

Appendix 2: Preparation PCRS11 medium

1. To 1L of H_2O, add 33,33g of Red Sea Salt
2. Dissolve by shake (20min on agitator)
3. Heat seawater during 20min at 100°C
4. Under hood, t o water, add these nutriments beforehand autoclaved (except vitamin) :

Quantity	*Compound*	*Final concentration*
1.0 mL	Hepes-NaOH 1M (ph 7,5) *(See receipe below)*	1mM
1.0 mL	Na_2-EDTA/$FeCl_3$ *(See receipe below)*	8µM
1.0 mL	Sodium Phosphate ($NaPO_4$) 50mM (pH 7,5) *(See receipe below)*	50µM
1.0 mL	Ammonium Sulfate 400mM $(NH4)_2$-SO_4	400µM
0,1 mL	Trace metals "Gaffron+Se" *(See receipe below)*	
0.1 mL	Cyanocobalamin 10mg/L (Vit. B12)	1µg/L

5. Filter the medium on 0,2microns

Hepes-NaOH 1M

To 250mL of H_20, add gradually 119,15g of Hepes. Adjust pH at 7,5 and complete the volume at 500mL. Store in refrigerator.

Na_2-EDTA/$FeCl_3$

- To 40mL of HCl 0,1N, add gradually 1,080g of $FeCl_3$
- To 40mL of NaOH 0,1N, add gradually 1,488g of Na_2-EDTA
- Mix both solutions
- Complete final volume to 2L of sterile water
- Store in refrigerator

Sodium Phosphate

- Prepare two solutions :
  - Monosodium dihydrogen phosphate (NaH_2PO_4) at 50mM (6g in 1L)
  - Disodium hydrogen phosphate (Na_2HPo_4) at 50mM (3,55g in 500mL)
- Make an equimolar mixture of this two solutions and adjust the pH at 7,5

Trace metals "Gaffron+Se"

To 500mL of H_20, add gradually these nutriments :

Quantity (mg/L)	Compound	Final concentration in media (nM)
186	Boric acid (H_3BO_3)	150
101	Manganese (II) Sulfate Monohydrate ($MnSO_4.H_2O$)	30
1,98	Sodium Tungstate dihydrate ($Na_2WO_4.2H_20$)	0,3
5,16	Ammonium molybdate tetrahydrate ($(NH_4)_6MO_7O_{24}.4H_2O$)	1,45
7,14	Potassium bromide (KBr)	3
4,98	Potassium iodide (KI)	1,5
17,25	Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$)	3
9,25	Cadium Nitrate ($Cd(NO_3)_2.4H_2O$)	1,5
8,76	Cobalt (II) Nitrate ($Co(NO_3)_2.6H_2O$)	1,5
7,5	Copper (II) Sulfate ($CuSO_4.5H_2O$)	1,5
7,1	Nickel Chloride ($NiCl_2.6H_2O$)	1,5
2,4	Chromium (III) Nitrate ($Cr(NO_3)_3.9H_2O$)	0,3
1,5	Vanadyl Sulfate Pentahydrate ($VOSO_4.5H_2O$)	0,3
28,4	Aluminium Potassium Sulfate ($KAl(SO_4)_2.12H_2O$)	3
3,3	Selenium (IV) Oxyde (SeO_2)	1,5

Complete the volume at 1L. Store in refrigerator.