



MaCuMBA

Marine Microorganisms: Cultivation Methods for Improving their
Biotechnological Applications

Project number: 311957

Start of the project (duration): August 1st, 2012 (48 months)

Collaborative Project
Seventh Framework Programme
Cooperation, KBBE

Deliverable D8.1 Automated system for isolation of single micro-organisms and their delivery into microbioreactors

Organisation name of lead contractor: HWU (partner 17)

Due date of deliverable: M36

Actual submission date: M37

Revision: V.1

Project co-funded by the European Commission within the Seventh Framework Programme (2007-2013)	
Dissemination Level	
PU Public	
PP Restricted to other programme participants (including the Commission Services)	
RE Restricted to a group specified by the consortium (including the Commission Services)	X
CO Confidential, only for members of the consortium (including the Commission Services)	

All rights reserved

This document may not be copied, reproduced or modified in whole or in part for any purpose without the written permission from the MaCuMBA Consortium. In addition to such written permission to copy, reproduce or modify this document in whole or part, an acknowledgement of the authors of the document and all applicable portions of the copyright must be clearly referenced.

List of reviewers

Issue	Date	Implemented by
v.1	7 Sept 2015	Gerard Markx

Indicate any document related to this deliverable (report, website, ppt etc) and give file name

** Please attach deliverable documents and any additional material if needed.*

Summary

Deliverable D8.1. Automated system for isolation of single micro-organisms and their delivery into microreactors

Objective(s): The objective of this deliverable was to develop an automated method for the isolation of microbial cells and their subsequent culture in microreactors.

Rationale:

Samples taken from the marine environments are highly heterogeneous, containing many different species. Unfortunately amongst those species there are many which are fastidious and abundant, whilst many of the species of interest are rare and difficult-to-culture. Selective separation of rare species from the other cells and their culture in isolation could help in achieving pure cultures of rare isolates.

Results

A compact optical tweezers system was designed and built. The system comprises an improved optical system for trapping and cell observation; a motorised mechanical stage (X, Y and Z); custom-built chips for cell isolation and culture (fabricated either in fused silica with ultrafast laser inscription and selective chemical etching (see Figure 1 and 2) or in PDMS with soft lithography (see Figure 3 and 4); a Labview image analysis programme for automated detection of cells and selection of cells to be isolated based on shape; and an automated system for cell movement along a predefined path.

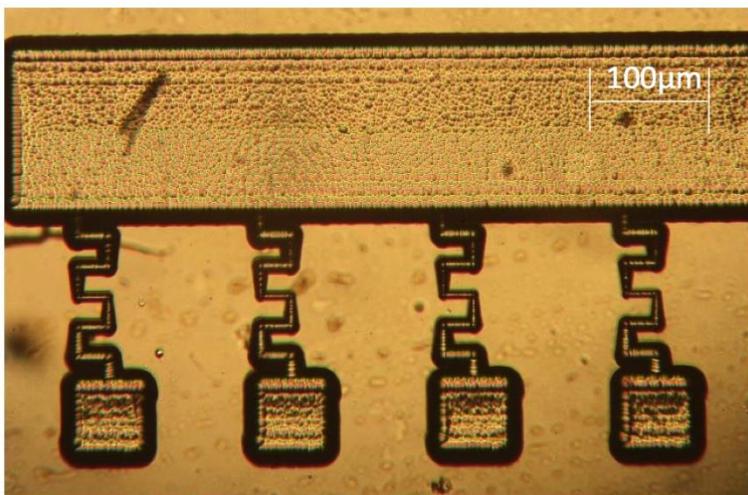


Figure 1: Chip made using ultrafast laser inscription and selective chemical etching.

The chips for cell culture included a main channel for the introduction of the cell mixture, and arrays of microchambers for cell culture connected to the main chamber through a narrow meandering

channel to prevent accidental introduction of cells into the microchamber and the escape of cells from them.

The setup was tested with model microorganisms. Yeast cells (*S. cerevisiae*) (Figure 2) and bacteria (*E. coli*, *B. subtilis*, *M. luteus*) could be isolated within these chips. A co-culture of a yeast cell and a bacterial cell was also established (Figure 3).

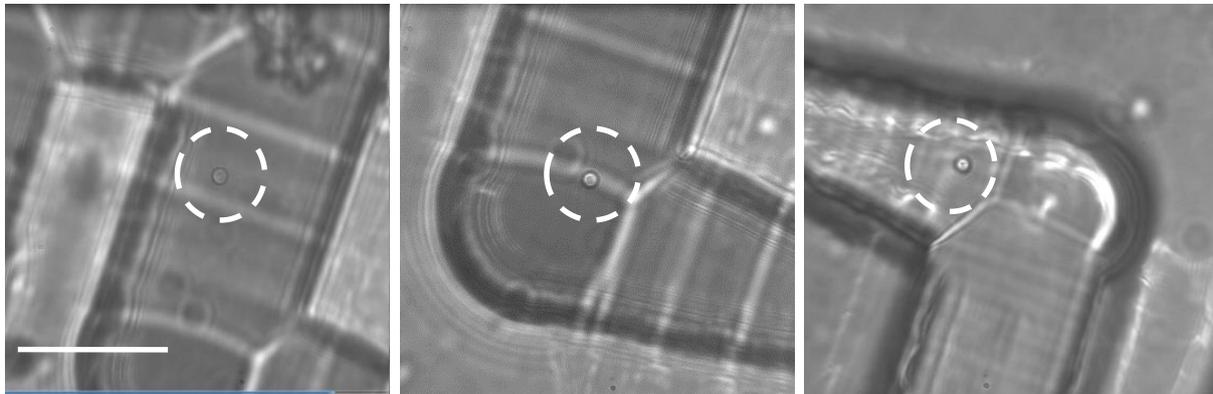


Figure 2: Yeast cell isolation through meandering channel in fused silica device.

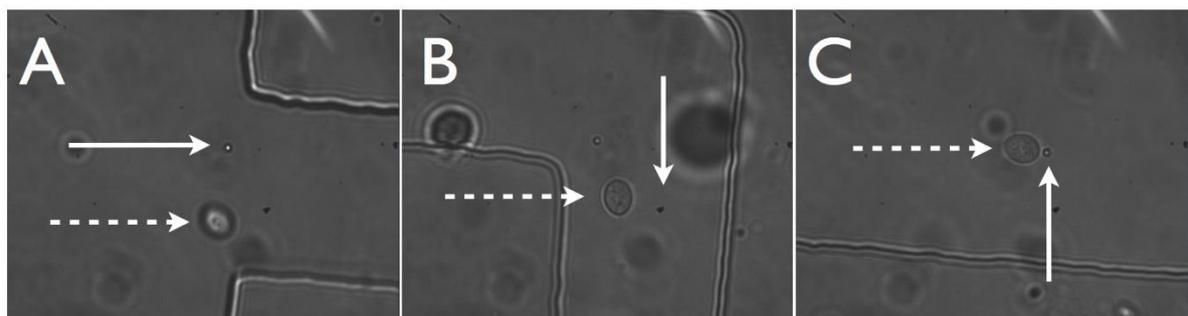


Figure 3: Manipulation of yeast and bacteria into isolation chamber in PDMS device. Solid line points to bacteria, dashed line to yeast. The cells are optically tweezed together from the main channel (A) through the meandering channel (B) and deposited into the isolation chamber (C).

Partner(s) involved in Deliverable production:

Specify the list of MaCuMBA partners and other contributors that have worked on this Deliverable (just the name of the organisations, not the name of the persons).

NIOZ partner 1

Heriot-Watt University (HWU) partner 17

Protocols for the construction of the laser tweezer system

The automated stage is built using the following parts from Thor labs: three 12 mm Motorized Actuator, 3/8" Barrel Fitting (0.5 m cable) (one each for x, y and z control of sample position), three T-Cube DC Servo Motor Controllers, (one for each motorized actuator), T-Cube Controller Hub and Power Supply Unit and a Thor Labs 13 mm XYZ Translation Stage. The T cube controller Hub is connected to the laptop and LabVIEW can be used to control the motorized actuators.

A laser (785 nm, 250 mW Min, Butterfly Laser Diode, SM Fiber, FC/APC) is connected to a Compact Laser Diode/Temperature Controller with Butterfly Mount. The laser output is directed via two turning mirrors (\varnothing 1" Broadband Dielectric Mirror, 750-1100 nm), two lenses (N-BK7 Plano-Convex Lens, \varnothing 1", $f = 100.0$ mm, ARC: 650-1050 nm) and a dichroic mirror (Hot mirror, 1" diameter, 45 degrees AOI) into a microscope objective lens (100X Nikon Plan Fluorite Oil immersion objective, 1.3 NA, 0.2 mm WD). The optical elements are mounted using Thor Labs cage rods, cage plates and posts.

The sample is illuminated from below (critical, transmission illumination) by a Cold White Mounted High-Power LED, 1000 mA controlled using a T-Cube LED Driver, 1200 mA Max Drive Current that is also placed on the same T-Cube Controller Hub and Power Supply Unit as the motorised actuators. An Infinity-Corrected Tube Lens for Plan Fluorite Objectives is placed at the back aperture of the 100X Nikon lens and an image is focused onto the chip of a High Resolution USB2.0 CMOS Camera, (1280 x 1024, Monochrome).

A drop of Low Auto Fluorescence Immersion Oil is placed on the sample chip prior to imaging cells and tweezing.

Protocols for the construction of the isolation chambers

PDMS device

The mould for soft-lithography can be produced with standard photolithography of SU8 on Si-wafers. SU8 3005 can be used for making channels of a low depth (8 μ m), whilst SU8 20250 is used for creating higher depth channels (25 μ m). The channel designs are made in AutoCAD software (Figure 4).

Mould fabrication

The pre-treatment of the silicon wafers is the same for both moulds. The silicon wafers are submerged in a 5% Decon90 solution for 45 min in an ultrasonic bath before thoroughly rinsed with acetone, followed by isopropanol and water. After that, they are heated to 120°C for 60 min on a hotplate. While they are still hand warm when removed from the hotplate, SU8 3005 or SU8 2050 respectively are spin coated on the wafers. The spin speed for the thin SU8 (SU8 3005) is 1200 rpm (SU8 2050: 4500 rpm). To remove the solvent of the photoresist the wafer is placed on a hotplate @65 °C for 2 min and @95 °C for 5 min (SU8: @65 °C for 3 min and @95 °C for 6 min). The energy dose under the UV-aligner is adjusted to 200mJ/cm² (SU8 2050: 160mJ/cm²). Before developing the wafer in EC solvent, the photoresist is post-exposure baked @65 °C for 1 min and @95 °C for 2 min (SU8 2050: @65 °C for 1 min and @95 °C for 6 min).

Sigmacote is applied to the wafer surface to render all structures to hydrophobic, which will make it easier to peel off the PDMS mould later on.

PDMS fabrication

The two components of the Silgard 184 kit are mixed in a ratio 10:1 (elastomer to curing agent) and degassed inside a desiccator for 20 min to remove any air bubbles. The mixture is poured over the SU8 mould and after that the mould including the PDMS is put into the oven @65 °C for 2 h so that the PDMS is cured and solidified. After letting the wafer and PDMS cool down to room temperature the PDMS is carefully peeled off from the mould, cut into the correct size with a razor blade and inlet and outlet holes are punched with a 1.5mm (or 2mm) biopsy punch. Together with clean cover slips the PDMS pieces are exposed to oxygen plasma for 1 min. Immediately after the treatment the two pieces are brought into contact. This needs to be done very carefully and without any physical force as this might close the delicate channels of the PDMS chip. To ensure a good bonding quality the samples are heated up to 80 °C for 1 h in an oven.

Sample preparation

The device is injected via an input hole with 0.075% Tween 20 in LB. Cells are then injected into the device and the device is mounted on a microscope slide and examined under the optical tweezers microscope.

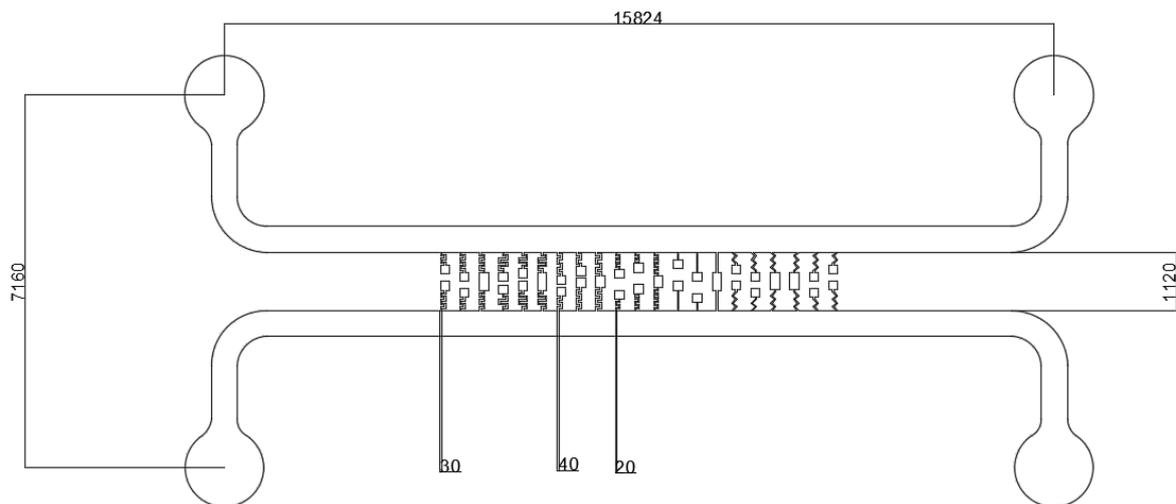


Figure 4: Chip design using AutoCAD (distances are in μm)

Fused silica device

Ultrafast laser inscription and selective chemical etching can be used to fabricate a microfluidic channel network, including micro-bioreactor chambers, on the surface of fused silica chips (Figure 1). A commercial Yb – doped master oscillator power amplifier fibre laser system (IMRA -FCPA μ Jewel D400) is used to deliver 1047 nm laser pulses at a repetition rate of 500 kHz with a pulse width of 358 fs. The laser pulses are focused inside a fused silica substrate using 0.4 NA (x20) aspheric objective lens. The translation of the substrate was controlled using Aerotech x-y-z air bearing stages. The chip design is then inscribed within the device by translating the x-y-z stage through the laserbeam focus in three dimensions. This direct laser writing process was then followed by selective chemical etching, where the sample substrate is etched using 5%HF in an ultrasonic bath. The HF etches out the laser-modified regions, creating the microfluidic channels and microbioreactors on the surface of the fused silica. The chip is injected with buffer and then the large reservoir is injected

with cells. A coverslip is placed on top and then a drop of immersion oil is added on top of the coverslip before imaging and tweezing.

Protocol for using the automated optical tweezers for cell isolation.

A Labview programme has been developed for cell isolation (automated or manual) using a laser tweezer system on pre-fabricated chips. The program can detect cells from the microscopic image captured by the camera and choose the best cell to be optically tweezed and isolated based on its shape. Alternatively, a user can choose the cell by observing the microscope image from the camera on a computer screen and manually selecting a cell using the laptop keys.

The programme is opened by loading the file “Automation for yeast.vi” into Labview.

Before launching the program verifies that’s everything is connected to the computer:

- The camera
- The hub for the stage controllers and LED illumination
- The laser

If the controllers do not respond to the programme:

- Stop the program
- Close LabVIEW
- Turn off the controllers alimentation and wait 5sec
- Turn it on
- Restart LabVIEW and the program

Cell detection and analysis (using LabVIEW acquisition and LabVIEW assistant)

The camera is detected automatically with LabVIEW acquisition. The brightness, contrast, gain and exposure of the camera can be modified in the “Image” Tab in the Front Panel (Figure 5). By default, the different values are set as: Brightness=0, Contrast=0, gain=0 and exposure=0.125. Changing these properties may enhance the images on the right of the front panel and may increase the quality of the detection.

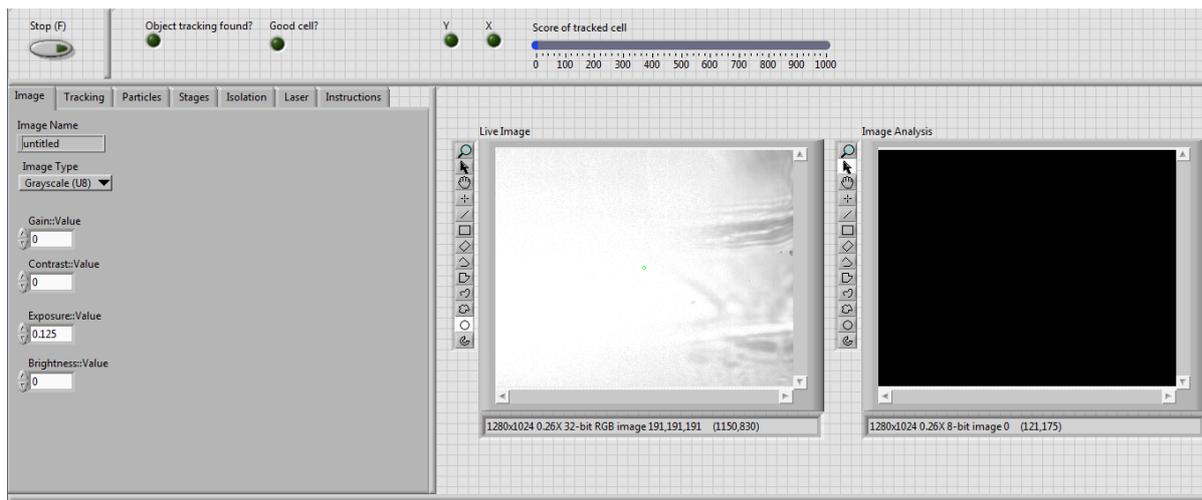


Figure 5: LabVIEW Front Panel; Image Tab

Image processing and analysis performed by the LabVIEW vision assistant. The software converts the image into a binary image in order to process it. It removes the background, the small particles and enhances the cells (they will appear in white on a black background on the right hand part of figure 5). The software counts the number of objects in the image and performs a series of measurements on each object; currently it measures area and the shape. This information is used to determine which of the objects is that of a cell shape is that of a cell. The data; number of cells, area and shape of each cell is presented in the Front Panel in the Tab named “Particles”. These settings cannot be changed from the Front Panel; however, they can be changed in the LabVIEW vision assistant software.

Cells can be tracked using a cell image, which is compared to the live image. If a cell is found with the same shape it will be framed into a red square. The tracking is performed whilst the cell is being optically tweezed to ensure that the cell is not lost from the optical trap. The tracking settings may be changed in the Front Panel in the tab “Tracking”; see figure 6.

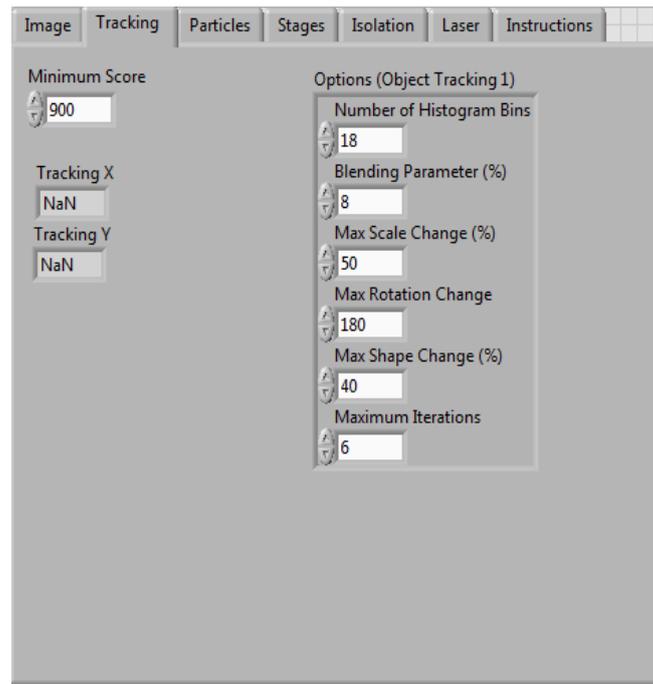


Figure 6: Tracking Tab in LabVIEW Front Panel

The settings that may be changed are:

-“Score”: a mark is attributed to the tracked cell, which corresponds to the similarity of the tracked live cell compare to the previous cell image. By default the value is 900; so if a cell has a score below 900 it will be marked as not found.

- “Number of Histogram Bins”: The more histograms there are, the more precise is the tracking. The default value is 18.

-“Blending parameters”: Between 0 and 100. 0 only the current ‘live’ image is analysed, 100 only the previous image is analysed. Default value is 8.

-“Max Scale Change”, “Max Rotation change”, “Max Shape Change”: Compare the cell from the live image to the previous saved cell image. Default values are: 50, 180, and 40.

-“Maximum iterations”: How many times the operation is repeated if no cell is found. Default is 6.

Tracking X and Y refer to the tracked cell’s coordinates (in pixels).

Stage control

You can control the stages manually using computer keys as follow:

- X stage: up and down keys.
- Y stage: left and right keys.
- Z stage: plus and minus keys.

You can also control the stage movement by placing the mouse cursor on the image and pressing the space bar. The stage will move and position the selected area to the centre of the image. Settings for the movement of the stage; velocity, acceleration, jogs size and the serial numbers of the stages can all be controlled from the ‘Stages’ Tab.

Automatic cell isolation

The isolation sequence is launched when P (for play) is pressed on the keyboard. A cell must be in the image centre. The laser turns on with a chosen power (this can be changed in the “Laser” Tab). The Z stage lowers to lift up the cell away from the surface of the sample (stage velocity and the acceleration can be changed in the “Stages” Tab). The X and Y stages then move in a pre-programmed pattern, depending on the chip design, to take the cell to the isolation chambers. (Note: the cell has to be at the isolation chamber entrance before beginning the pattern of movement.) The pattern can be changed in the “Isolation” Tab (Figure 7).

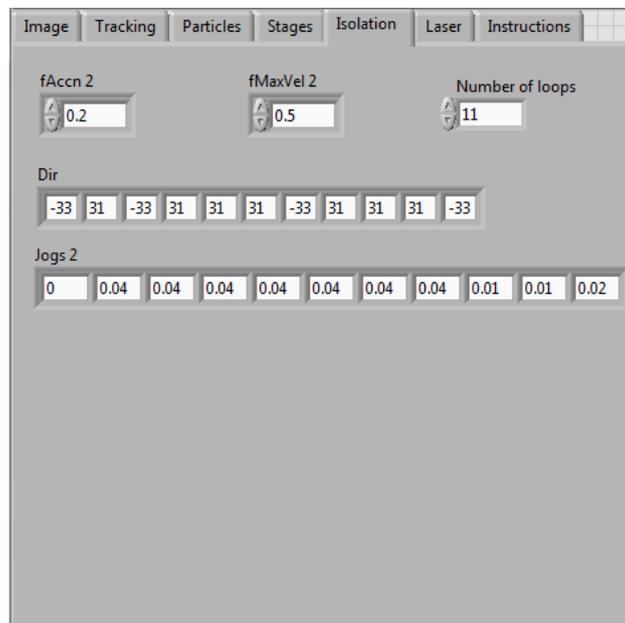


Figure 7: Isolation Tab in LabVIEW Front Panel

The number of loops means the number of changes in direction. The step size (in mm) can be entered (under Jogs 2) and the movement direction is given by ‘31’ or ‘-33’. Note that you have to begin with the X stage (the step size can be set at 0). Once the stages complete the pattern motion and have taken the cell to the chamber, the laser turns off and the stages return to the home position. The movement parameters (velocity and acceleration can be set in the “Isolation” Tab).

Manual control

Single cells can also be isolated manually using the computer keys. The laser can be manually turned on and off by pressing the Q and W key, respectively.