A new method for fungal genetics: flow cytometry of microencapsulated filamentous microcolonies

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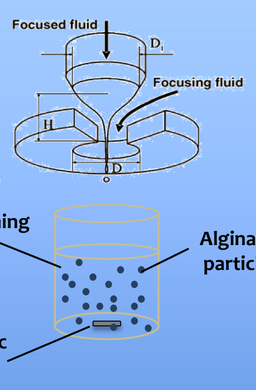
Microencapsulation technology

Focused fluid: alginic acid solution with sample of cells



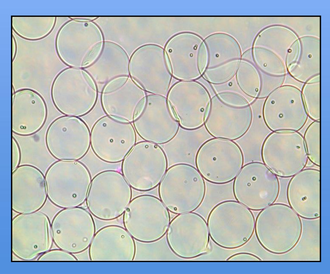
Focusing fluid: compressed air, N_2

Cellena® portable microencapsulator

Procedure:

1) **The sample is injected through a capillary feed tube.**

2) **The stationary jet breaks up by capillary instability into homogeneous droplets which gel in a continuously stirred calcium chloride solution at room temperature.**



Spherical size-monodisperse alginate microcapsules

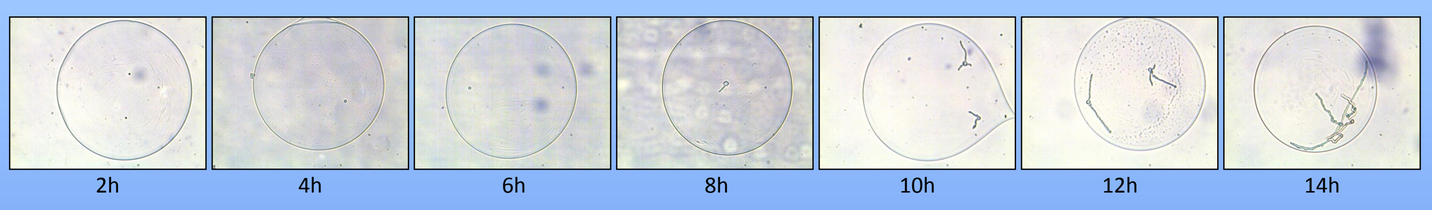
Abstract

Genetic analysis of non-filamentous microorganisms is facilitated by the isolation of consistent, well-defined colonies on solid media and the handling of individual cells by flow cytometry. In contrast, some filamentous fungi are hard to be analyzed using these procedures; in particular by flow cytometry. The combination of single spores microencapsulation and large particle flow cytometry is a possible alternative for the analysis of filamentous fungi. Microencapsulation allows the early detection of fungal growth by monitoring the development of hyphae from encapsulated individual spores. Mycelium proliferation inside the microcapsules can be detected using COPAS™ large particle flow cytometry.

Here we show the successful application of the Flow Focusing® technology to the microencapsulation of filamentous fungi in monodisperse alginate microspheres, using *Aspergillus* and *Trychoderma* as model systems. Using a Cellena® Flow Focusing microencapsulator, we managed to produce monodisperse microparticles containing individual spores and to develop microcolonies of these fungi upon germination in the appropriate conditions. Proliferation inside the particles was monitored by microscopy and large particle flow cytometry without requiring fluorescent labeling. Sterility was preserved during the microencapsulation procedure, preventing undesired contaminations. Conditional mutants were utilized to demonstrate the feasibility of the method. This procedure allows for the handling, screening and analysis of clonal colonies in liquid culture. Examples of applications are provided.

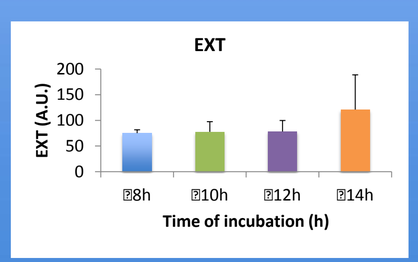
Monitoring the proliferation of *Trichoderma reseei* spores by optical microscopy and flow cytometry

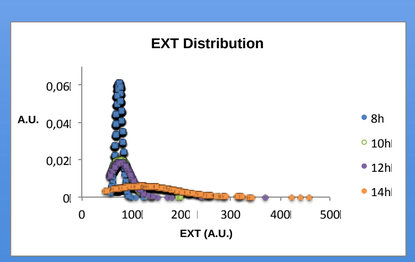
In this test the spores were encapsulated in 400µm, 1,66% alginate capsules . After encapsulation the beads were incubated in shaking flasks and samples were recovered after 2h, 4h, 6h, 8h, 10h, 12h and 14h of incubation. Aliquots were analyzed by COPAS SELECT flow cytometry allowing the measurement of different optical parameters: size (TOF), optical density (EXT), green self-fluorescence and red self-fluorescence signals.



Pictures of encapsulated spores during germination at different times points.

The germination of spores is associated with an increase in density as measured by the COPAS system. These measurements are represented in the next graphs showing the increase of EXT over time (left) and the EXT distribution within the bead (right).





Graphs showing the increase in EXT (scattered light) and the distribution of EXT during time of incubation.

Optical parameters analyzed by COPAS™.

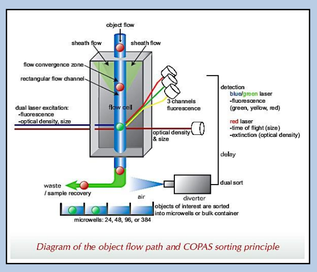
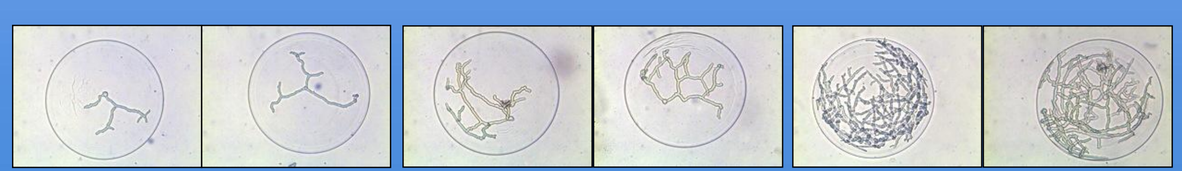


Diagram of the object flow path and COPAS sorting principle

COPAS™ instruments allow to automate the process of sorting large (20-1,500 µm) particles in a continuously flowing stream at a rate of 10-50 objects/second. Using object size (TOF), optical density (EXT), and intensity of fluorescent markers as sorting criteria, selected objects in this size range, can be dispensed without any harm in multi-well plates for further analysis.

To optimize the encapsulation further, it was evaluated whether encapsulation of spores using higher percentages of alginate (3%) would allow the mycelia to grow in a more compact way. This could also increase the incubation time before the mycelia grow outside the capsule. Pictures of growing mycelia were taken after 14h, 16h and 18h of growth.

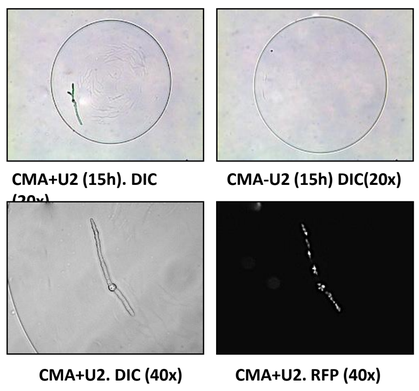


Genetic screenings of *Aspergillus nidulans* using encapsulation

I. Encapsulation of *Aspergillus*

Encapsulation of filamentous fungi can be used for genetic screenings using large particle flow cytometry of single spores. Encapsulation allows to screen for growth / no growth and/or fluorescence (see example 1 with the auxotrohic mutant *pyrG89*). This screening can be extended to search for conditional mutants (see example 2 with the thermosensitive allele of *nimX*^{F233L}*).*

A. nidulans* HA344: H1::RFP *pyrG89



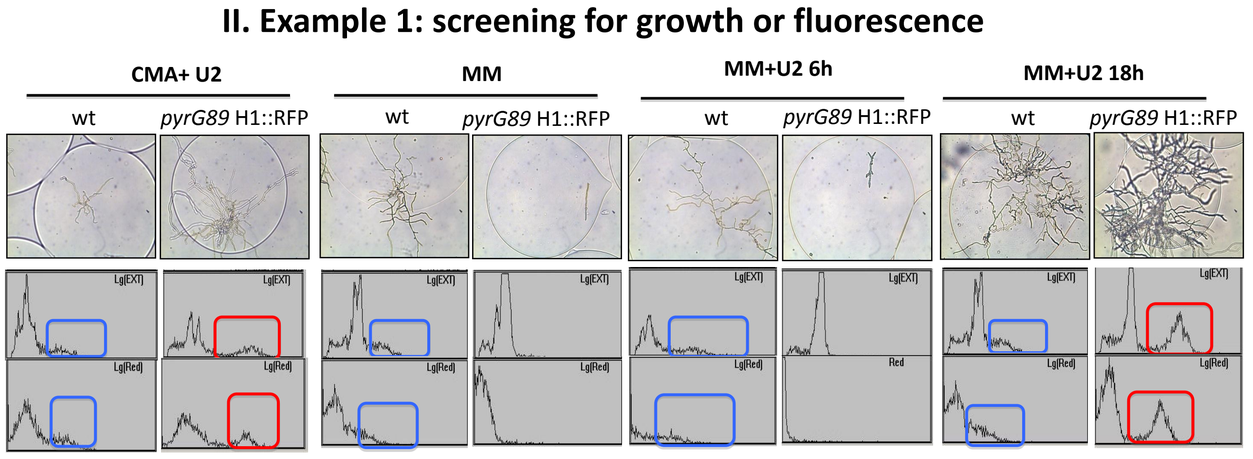
CMA+U2 (15h). DIC (20x)

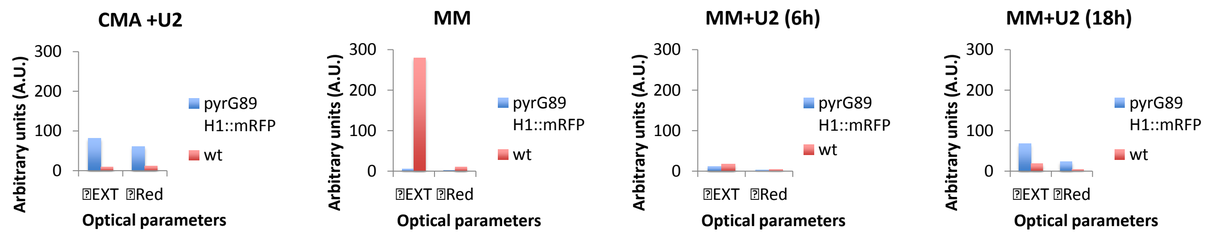
CMA-U2 (15h) DIC(20x)

CMA+U2. DIC (40x)

CMA+U2. RFP (40x)

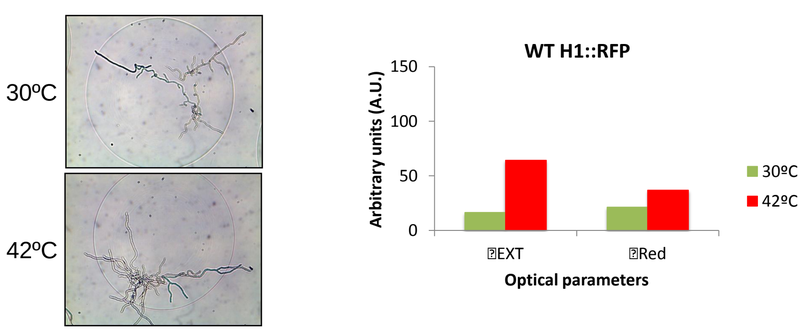
II. Example 1: screening for growth or fluorescence





III. Example 2: screening for conditional mutants using thermosensitive alleles

WT H1::RFP



***nimX*^{F233L} H1::GFP**