Developing a novel method for the screening of fungal germinated spores using hydrogel microencapsulation and large particle flow cytometry.

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Objective

Evaluation of large particle flow cytometry for the screening of encapsulated germinated fungal spores to select for colonies displaying unique characteristics.

Introduction

Genetic analysis of filamentous fungi and characterization of libraries transformed in fungal spores are tedious and time consuming endeavours. These processes usually require several culturing steps and manual manipulations to obtain clonal colonies. Here we present the development of a novel method to screen these libraries using hydrogel microencapsulation and large particle flow cytometry.

A variety of cell types can be enclosed in alginate, maintaining their morphology and function; they proliferate, form cell clusters, and even lay down extracellular matrix components. Furthermore, the cells can be encapsulated in small particles that can then be handled, characterized and analyzed. These features make alginate a widely acceptable material for three-dimensional scaffolds of live cells.

We have encapsulated single *Trichoderma reseei* spores using Cellena® flow focussing® technology and then we have analyzed the encapsulated germinated spores on a COPAS™ large particle flow cytometer (Union Biometrica, Inc). Cellena technology allows encapsulating spores in predominantly monodisperse populations of spherical microparticles of the selected size, without any decrease in viability. The strength of flow cytometric sorting is the ability to follow the selection in real time and to easily isolate the spores/colonies that display desired characteristics. Proliferation of the mycelia within the particles can be monitored without any labelling (optical density measurement). Furthermore, fluorescent markers can be used to select for colonies with higher production levels of a particular tagged protein. Clonality can be established, and then maintained, during subsequent manipulation by individually dispersing the encapsulated germinated spores onto a receptacle of choice for further downstream characterization.

The COPAS instruments automate the analysis, sorting, and dispensing of "large" objects such as viable small model organisms, seeds, beads, and particles, measuring the object size (TOF), optical density (EXT), and the intensity of fluorescent markers. Once analyzed, objects are sorted according to user selectable criteria, and then may be dispensed into stationary bulk receptacles or multi-well plates for high throughput screening. The COPAS instruments have been proven to analyze and sort large objects with a higher speed and precision than present manual techniques. By automating the current time consuming manual processes, the time required for experiments is dramatically reduced, human error is eliminated, and new experiments that previously could not be considered are now possible.

To avoid damaging or changing the beads, a gentle pneumatic device located after the flow cell is used for sorting and therefore makes the instrument suitable for handling very sensitive samples (Figure 1). The fluid pressures of the instrument are also significantly lower than those of traditional flow cytometers, resulting in lower shear forces and less damage to samples of interest.

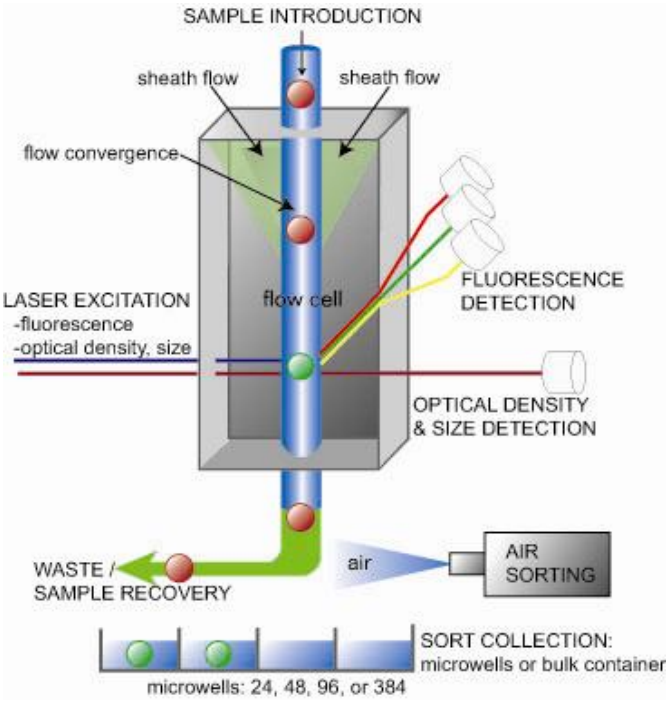

Figure 1. Analysis of beads inside the flow cell. Beads are carried through the flow cell by a liquid stream while their physical properties are being measured. Convergence of the sheath and sample fluid allows "hydrodynamic focusing" of the beads, directing them to go through the center of the flow cell. Inside the flow cell objects are illuminated by two low energy lasers that measure the object's optical properties of: Size, Optical Density, and Fluorescence. Those objects meeting sort criteria are permitted to drop into a sort collection vessel, while those that do not are diverted to waste recovery using a pneumatic sorting device.

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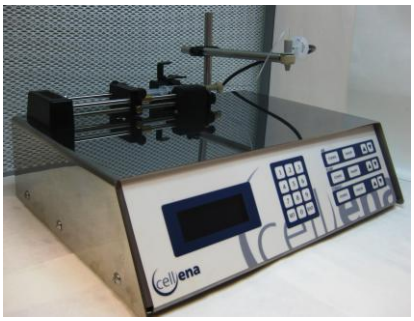
Materials and Methods

A COPAS SELECT™ flow cytometer (500 micron flow cell; Union Biometrica, Inc) equipped with two low energy lasers was used. A red diode laser (670 nm) to measure the diameter and the optical density of the bead, and a multi-line laser was used to excite any fluorophores. Based on the measured optical parameters (size, optical density, and fluorescence) the operator can then set gated regions for sorting and collecting the population of interest into multi-well microtiter plates or stationary receptacles.

For encapsulation of the spores Cellena® Flow Focussing® technology was used, which can generate smaller particles than other encapsulation technologies and allows for a high particle production rate per orifice, without clogging. Microparticles produced by the Cellena techonology are predominantly monodispersed and specific in size, which can be set in the 50 µm - 1 mm range, depending of the nozzle size. These nozzles are designed to be single-use and can be replaced easily, avoiding problems of microbial contamination. The nozzle is part of the capsule which, during encapsulation, contains alginate, the sample (a spore) and low concentration of sample medium. Figure 2 shows the Cellena encapsulation device and a schematic overview of the Flow Focusing encapsulation technology (A.M. Gañán-Calvo en at. International Journal of Pharmaceutics. 2006).



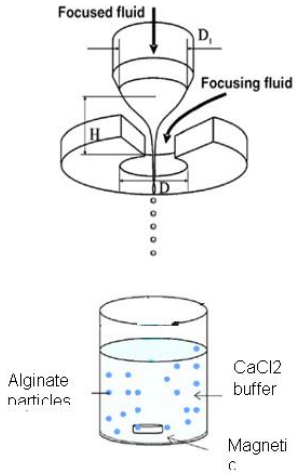


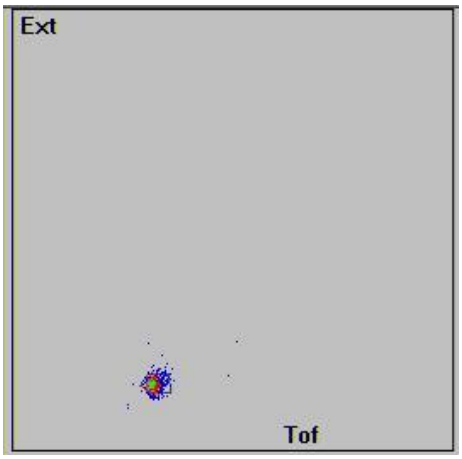
Figure 2. Cellena encapsulation device and schematic overview of encapsulation technology.

For this proof of principle the spores of the fungi *Trichoderma reseei* were encapsulated. Three different dilutions of Trichoderma spores were used for encapsulation in order to find the optimal dilution to generate 1 spore/bead. In this test the spores were encapsulated in 400 micron alginate capsules.

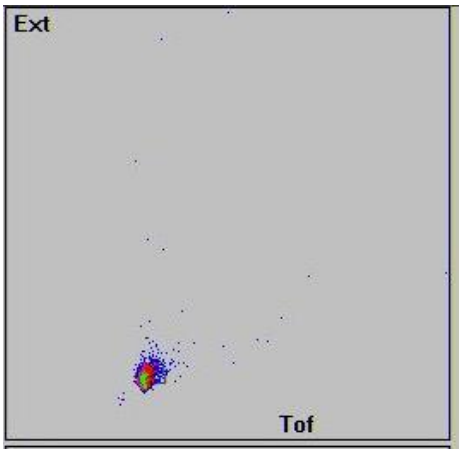
After encapsulation the beads were incubated in shaking flasks and aliquots were sampled after 2h, 4h, 6h, 8h, 10h, 12h and 14h of incubation. The aliquots

were analyzed on the COPAS SELECT flow cytometer and images from the dispensed beads were taken.

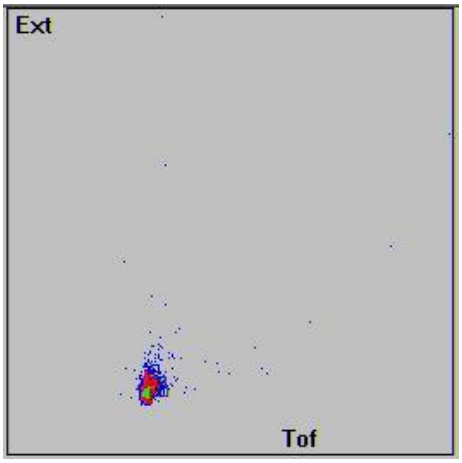
The beads were transferred to the sample cup and analyzed. They were selected based on their size (TOF) and optical density (EXT) and then sorted into a receptacle for microscopic verification and imaging. Figure 3 shows the dual parameter dot plots TOF vs. EXT for empty beads, 10h, 12h and 14h of incubation. After 14h incubation a clear increase in EXT is visible because this corresponds with the time that germination occurs and mycelia start forming.



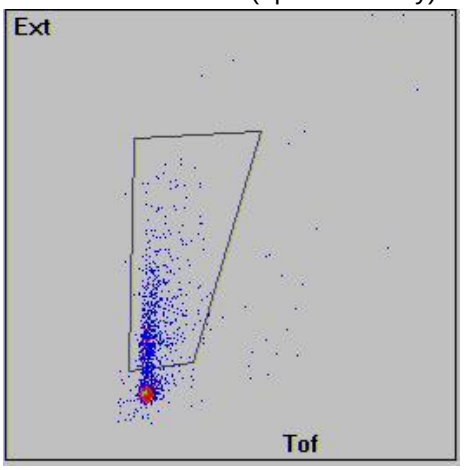
Empty beads (negative control)



10h incubation. Germination started and increase in EXT (optical density) detected



12h incubation



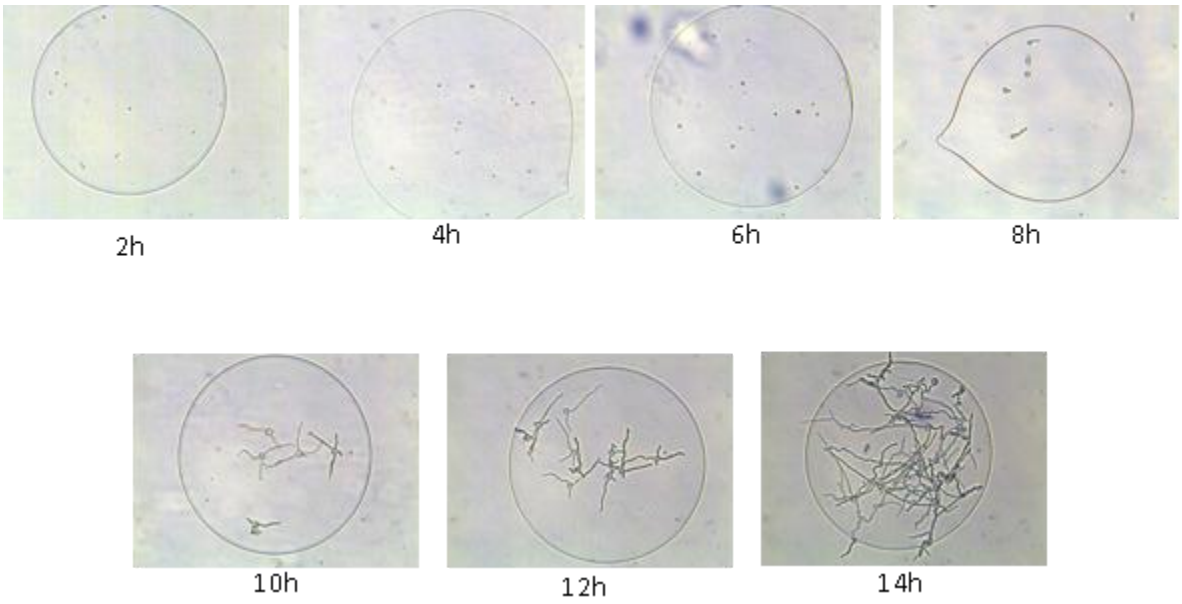
14h incubation and sharp increase in mycelia growth (reflected in increase Ext value).

Figure 3. Dot plots of encapsulated beads at different times of incubation (COPAS data). TOF ~ size and EXT ~ optical density. The dot plots allow for gating for a specific subpopulation of beads (eg.a gate was drawn around the population of beads where germination occurred, lower right dot plot).

The top left panel shows empty beads. The top right and the lower panels show germination of the spores after 10h, 12h and 14h. Between 12h and 14h there is a sharp increase in EXT levels which corresponds with growth of the mycelia. A stringent gating can be set around those beads having the highest density of mycelia.

Below a series of photo micrographs are shown for every sample dilution and analyzed time point (Figure 4). The alginate is transparent making the spores and mycelia visible under a standard stereomicroscope.

Pictures of the High Spore Density encapsulation and germination over time.



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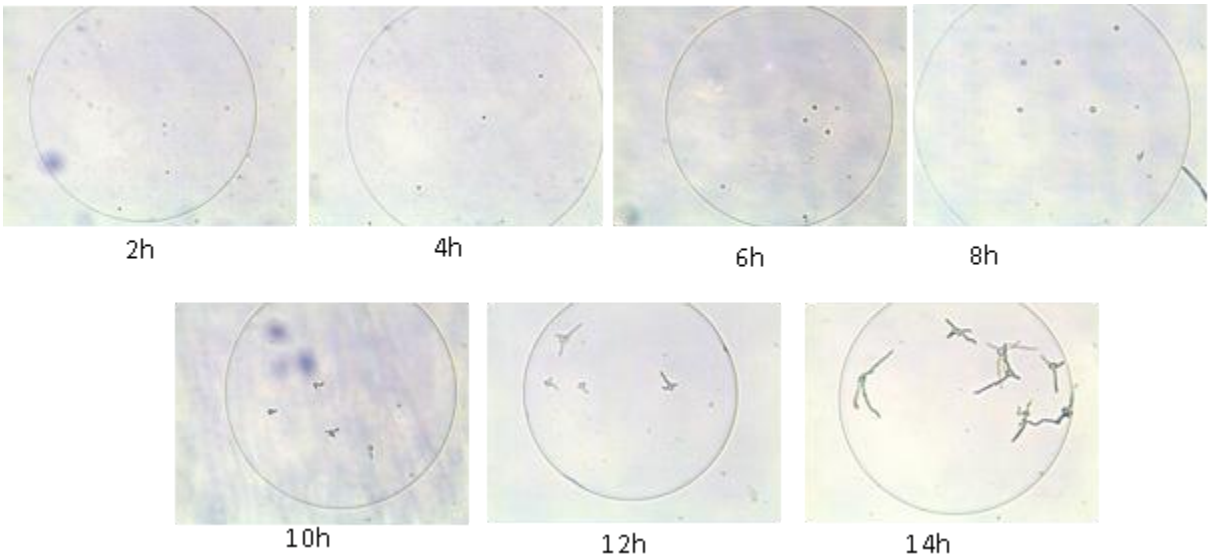
Pictures of the Medium Spore Density encapsulation and germination over time.



Pictures of the Low Spore Density encapsulation and germination over time.

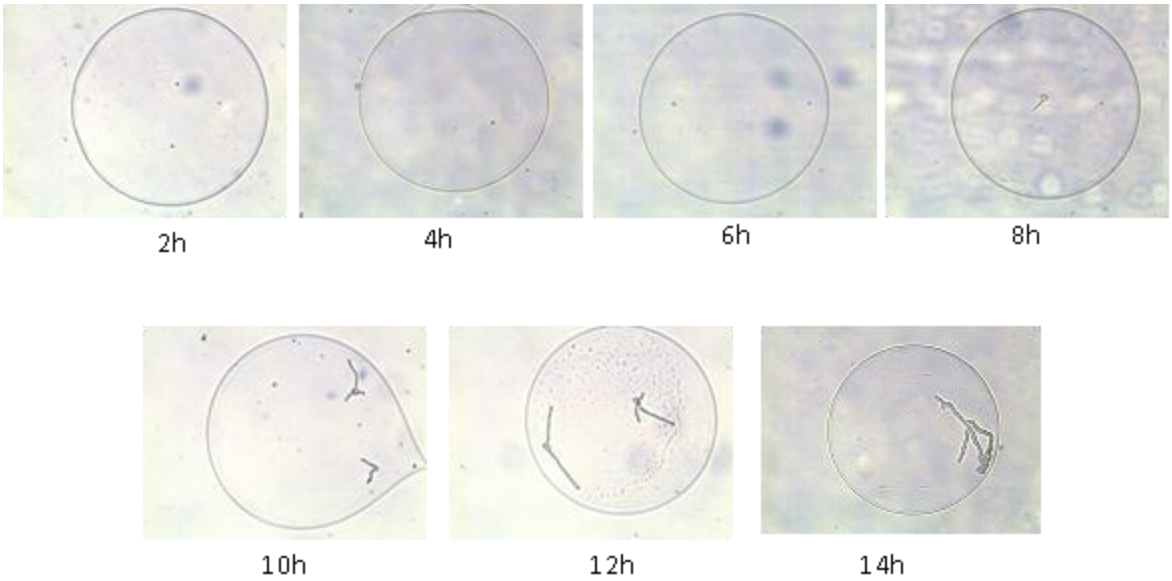


Figure 4. Pictures of encapsulated spores (High, Medium and Low density) at different times of incubation. Particles were formed with 1.6 % alginate.

The Low Spore Density encapsulation gave the best result to obtain 1 spore/bead. Some beads do contain more than one spore but the proportion of microparticles containing more than one spore can be minimized by performing the adequate dilution before encapsulating. Below (Figure 5) are more pictures given of germinated spores in the beads (14h incubation).

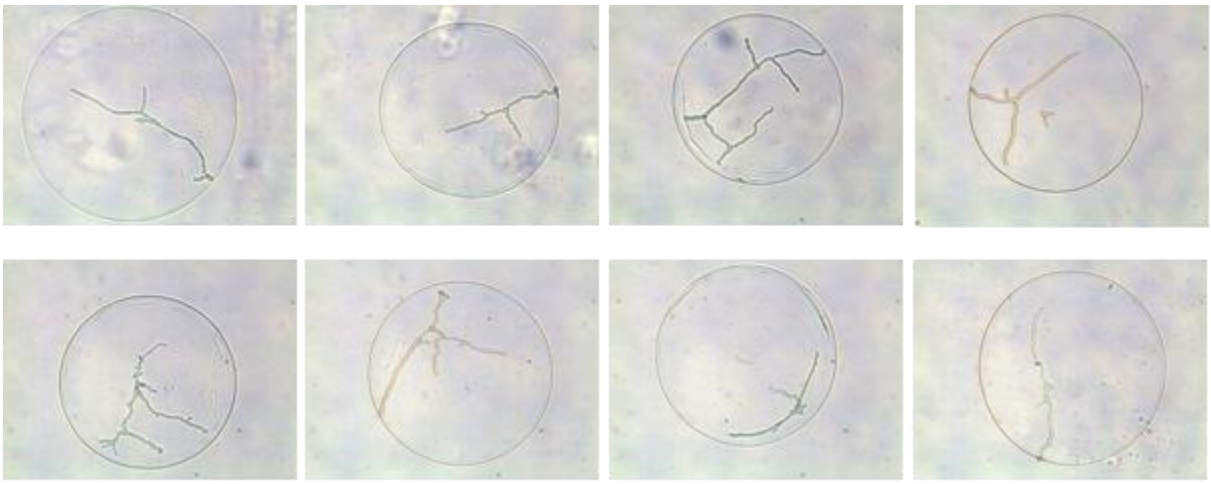


Figure 5. Germinated spores in beads that contain 1 spore/bead. Particles were formed with 1.6 % alginate.

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

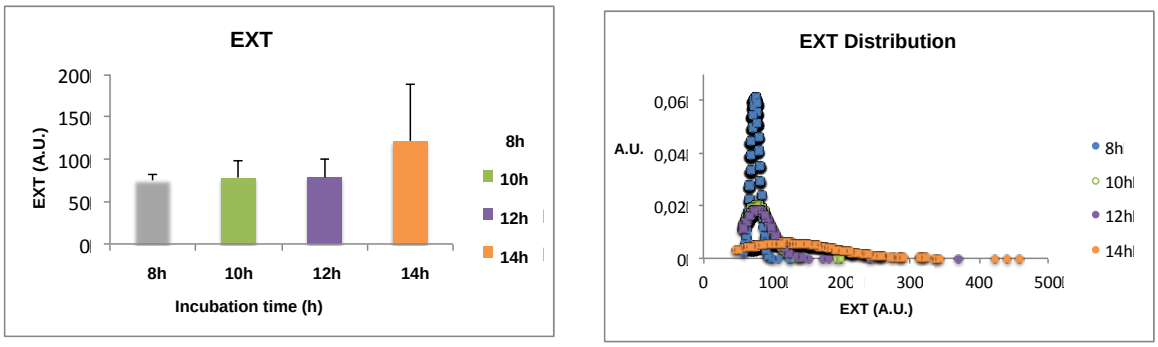


Figure 6. Graphs depicting the increase in Ext (~ optical density) and the distribution of EXT during time of incubation.

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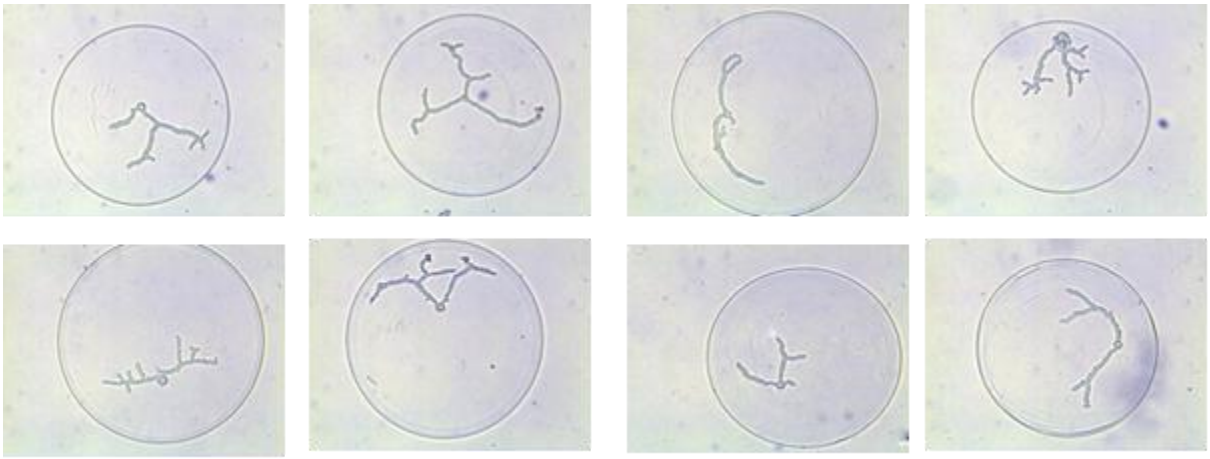
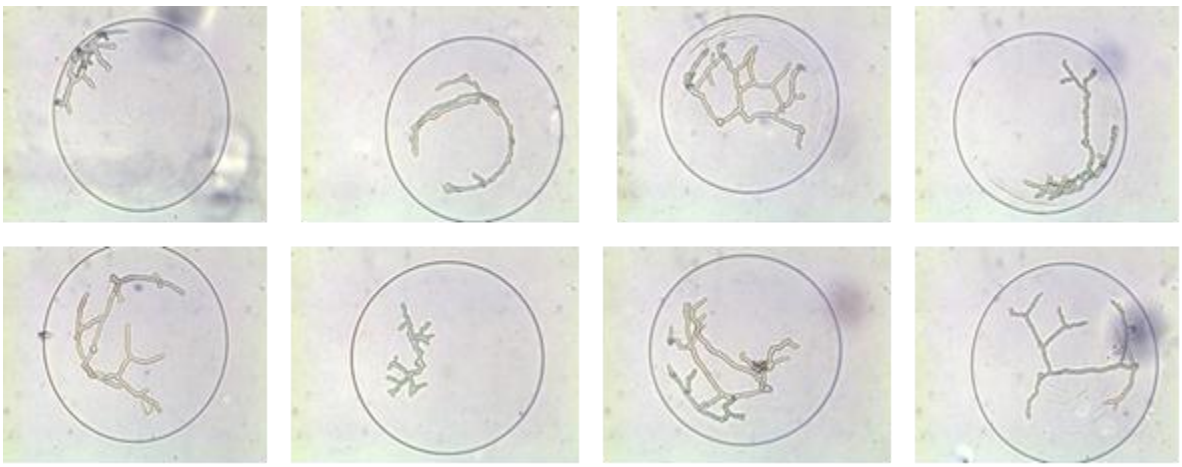
Graphical representation of mycelia growth in the capsule over incubation time is depicted in Figure 6. The longer the incubation the more dense the beads are and the greater the density distribution over the whole population (most beads are empty).

To optimize the encapsulation further it was evaluated whether encapsulation in higher percentages of alginate (3% instead of 1.6%) would allow the mycelia to grow in a more compact and dense way (low dilution of spores). This could also increase the incubation time before the mycelia grow outside the bead barrier. Pictures of germinated spores after 14h, 16h and 18h in 3% alginate beads are shown below (Figure 7).



14h incubation – 1 spore / bead



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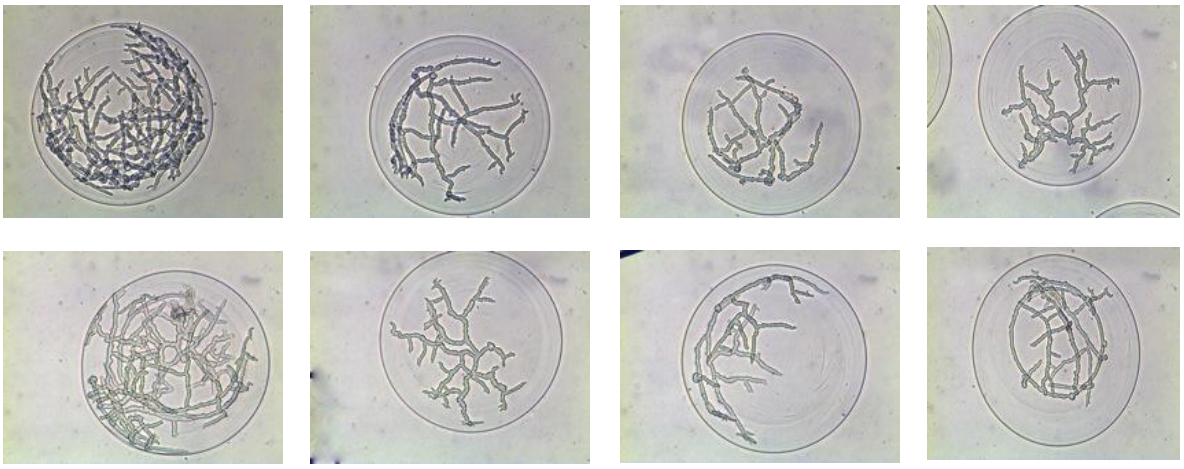
16h incubation – 1 spore / bead



18h incubation – 1 spore / bead

Figure 7. Germinated spores in 3% alginate microparticles. Dilution was set to maximize the proportion of beads containing 1 spore.

Using higher percentages of alginate to encapsulate the spores, the mycelia grow in a more compact way and incubation time can be increased to 16-18h. Coating materials like chitosan are available to retain the mycelia even more within the beads without reducing nutrient diffusion inside the bead.

Conclusion

These experiments demonstrate that the COPAS SELECT may be used to analyze and sort germinated fungal spores like Trichoderma reseei spores. The instrument can analyze and accurately select and sort for spores with larger mycelia from a complex mixture of varying mycelia densities. This automated analysis and sorting process is gentle and does not influence viability of these colonies.

COPAS large particle analysis instrument brings the method of flow cytometry to the analysis and sorting of germinated fungal spores not normally amenable to analysis on conventional single-cell flow cytometers. The combination of this instrument with Cellena Flow Focussing microencapsulation brings the advantages of flow cytometry – individual interrogation of each object in the sample, statistically meaningful data, large unbiased datasets, and multiparametric analysis – to the genetic analysis of filamentous fungi and the selection of transformed libraries in fungal spores.

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