Picodroplet Mass Spectrometry for Miniaturized High Throughput Analysis of Synthetic Biology Microbial Clones

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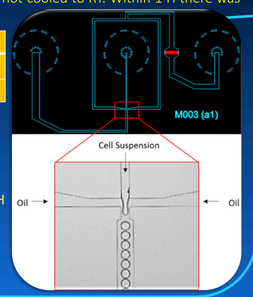
Introduction

We report the progress towards a high throughput, label-free single cell analysis method for characterizing synthetic biology clone libraries. Recent advances in DNA assembly have greatly improved a synthetic biologist's ability to design and efficiently build multiple gene pathway libraries in a combinatorial fashion. The large number of strains in these libraries leads to an urgent demand on high throughput analysis to help discover high-performance strains. Mass Spectrometry (MS) measures native molecules (*i.e.* is label-free) and yields comprehensive metabolic profiles of the presence, absence and concentration of multiple, specific molecules, but is relatively slow and low throughput. A current gold standard method, Agilent's RapidFire 360 system, can screen 1 sample every 7-9 seconds, which equates to 10-12 k samples/24 hour day. One of the major drawbacks of microtitre plate (MTP) based screening is the high volumes of reagents required for phenotypic screening of large synthetic biology libraries. Picodroplets are surfactant-stabilised water droplets in fluorous oil, 1-999 pL in size, and enable monoclonal compartmentalisation of cell library members. Use of 300 pL water picodroplets represents a 10,000-fold decrease in the sample volume required over MTP based screening, allowing proportional cost savings in reagents and media. Sphere Fluidics has recently developed a high-throughput ESI-MS system based using its proprietary picodroplet technology [1], which can test up to 200,000 biomolecular samples/day. With the development of this tool, synthetic biologists will now be able to truly fulfil the Design – Build – Test – Analyse cycle. Thus, they can discover the best engineered clones for their specific purpose; *e.g.* green manufacture of chemical feedstocks, biofuels, high value chemicals, enzymes, food additives, colourants, fragrances, plant protection organisms and new novel medicines . An overview of picodroplet workflows for synthetic biology is outlined below.

Encapsulation of bacteria in Picodroplets

In order to control the input bacterial encapsulation, it was found necessary to chill the bacteria (O.D. 0.2) at 7°C and then store at RT. The O.D. was monitored over 2.5 H and compared to a sample not cooled to RT. Within 1 H there was essentially no significant bacterial proliferation observed, see table below:

OD	0 min	5 min	10 min	15 min	20 min	60min	90 min	270 min
Room Temperature	0.2	0.21	0.2	0.2	0.20	0.22	0.24	0.43
7 °C	0.2	0.21	0.2	0.21	0.21	0.21	0.22	0.22



This means that as long as picodroplet generation is carried out in less than 1H there is a low probability that the bacteria can proliferate. Using the picodroplet generation biochip, see right, the used flow rates and generation statistics are shown below:

3% Pico-Surf™ 1 in Novec7500 at 3950 µL/H; Bacteria in growth media 200 µL/H

Picodroplet generation frequency = 1852 Hz (RT, 6.67 x10^6/H)

Therefore it will take only 18 minutes to make 2 million picodroplets, which is The minimum number required for the complete workflow. See how this number was derived in the next section on Poisson statistics (see right).

Single Cell Encapsulation in Picodroplets using Poisson Statistics

Describes the probability of events happening in a that the average rate (λ) of the event is known and independent of the previous even set period of time or space. Assuming that the average rate (λ) of the event is known and is independent of the previous event, see E1.

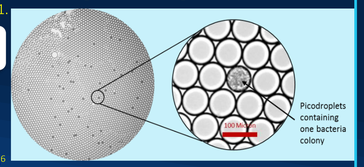
[E1] Probability = $f(\kappa;\lambda) = \Pr(X = \kappa) = \frac{\lambda^{\kappa} e^{-\lambda}}{\kappa!}$

K = Number of bacteria/picodroplet (0,1,2,3... *etc.*)

λ = Number of Bacteria (CFU / mL) / Number of picodroplets / mL

Number of picodroplets / mL = $\frac{0.001\ \text{L}}{3.0 \times 10^{-10}\ \text{L}} = 3.33 \times 10^{6}$

Therefore, if the volume of the picodroplet is defined at 300 pL, Then the only parameter that can be varied to alter the level of picodroplet occupancy is the value of the input level of the bacterial cfu. See the image opposite.

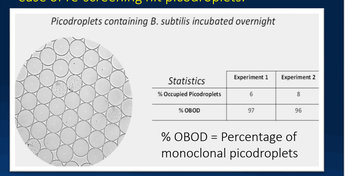


Note that there is some shrinkage of the picodroplets containing bacteria compared to empty picodroplets. This is likely due to production of bacterial metabolites affecting diffusion.

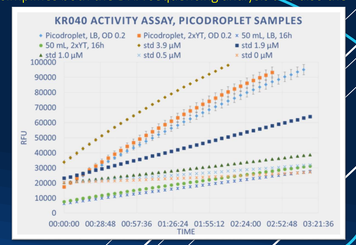
To screen 100,000 clones at a rate of 5% encapsulation means that there are 1.9 million empty picodroplets that need to be removed.

Design and build model organisms that express a ketoreductase

Here we found that *B. subtilis* with the appropriate amino acid leader sequences allowed ketoreductase (KRED) enzyme to be excreted into the supernatant. This means that at the assay can be carried out in the supernatant of the picodroplet, avoiding the need to lyse the cells. This was important as ideally we want to retain live bacteria from the ESI-MS biochip; as this simplifies both the DNA sequencing analysis and also the ease of re-screening hit picodroplets.

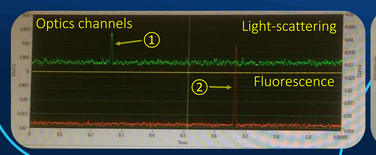


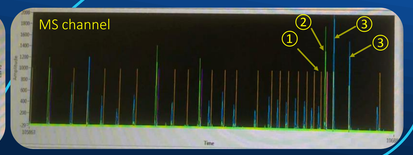
KRED activities were measured and exhibited a concentration 2-4 µM of KRED in the picodroplet supernatant. See graph opposite.



Optical & MS Coding Strategy & Software

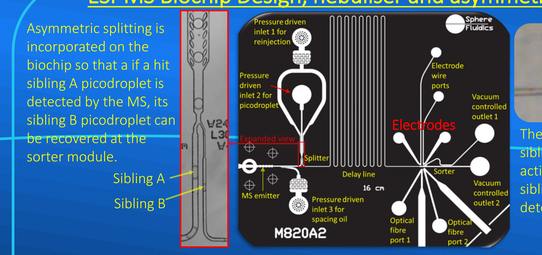
As all bacterial sibling B picodroplets appear optical identical at the sorting junction, there is a need to create a unique random coding strategy. We achieve this by adding about 10% Qdot 585 containing fluorescent picodroplets, called "zero"-picodroplets, to the bacterial picodroplets. These "zero" picodroplets also contain a unique mass tag to differentiate them in the mass spectrometer from the bacterial ones. Gentle mixing of the two populations of picodroplets creates a random mixture, which is then reinjected onto the MS biochip. The bacterial picodroplets will exhibit the masses from the KRED reaction, the ketone starting material and the alcohol product. The software allows up to eight different extracted ion chromatograms (EICs) to be analysed by the software. To define a hit the user selects an EIC threshold for the product ion. The software then compares each picodroplet signal's: the MS EICs and its optical signals; these are deconvoluted and coded accordingly as either: ① a bacterial picodroplet where the product EIC is below the threshold (coded brown), ② a "zero" picodroplet (coded green) or ③ a "hit" bacterial picodroplet, where the product EIC is above the threshold (coded blue) in MS window, see below right. The sibling A MS signals arrive approximately 1 second before the sibling B picodroplets arrive at the sorting junction. The coding system is the number of bacterial picodroplets after each "zero" picodroplet. When the next zero picodroplet arrives it resets the clock and it starts counting the number of bacterial picodroplets. Thus there are two integer strings: MS channel & optics channel; when these two integer strings match the software is synchronised and it will sort the correct hit sibling B picodroplet at the sorting junction. These Sibling B picodroplets approximately 60 pL in size should contain around 200-400 bacteria, enough to start a new colony for rescreening with more precision, e.g. HPLC MS.





ESI-MS Biochip Design, nebuliser and asymmetric splitting

Asymmetric splitting is incorporated on the biochip so that a if a hit sibling A picodroplet is detected by the MS, its sibling B picodroplet can be recovered at the sorter module.

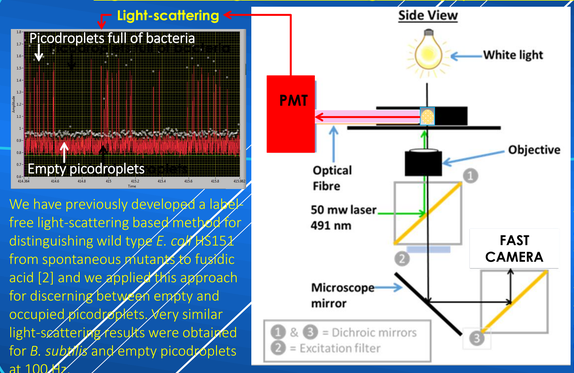


The image above shows a sibling B picodroplet being actively sorted, following a hit sibling A picodroplet being detected by the ESI-MS.

We chose to use a PerkinElmer AxION 2 ESI TOF mass spectrometer due to its high scan rate (100 Hz) and pulse trap technology. For this proof of concept experiment we utilised the standard PerkinElmer AxION 2 HPLC MS nebuliser with a custom capillary emitter (I = 128 mm) to connect to the MS biochip. See picture below.

This stainless steel capillary has been replaced with a gold coated plastic version.

Light Scattering based sorting of empty from occupied picodroplets

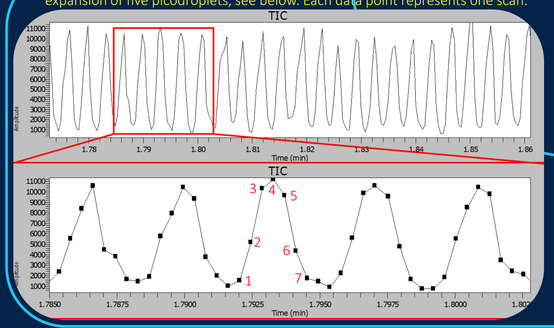


We have previously developed a label-free light-scattering based method for distinguishing wild type *E. coli* MG1521 from spontaneous mutants to fusidic acid [2] and we applied this approach for discerning between empty and occupied picodroplets. Very similar light-scattering results were obtained for *B. subtilis* and empty picodroplets at 100 Hz.

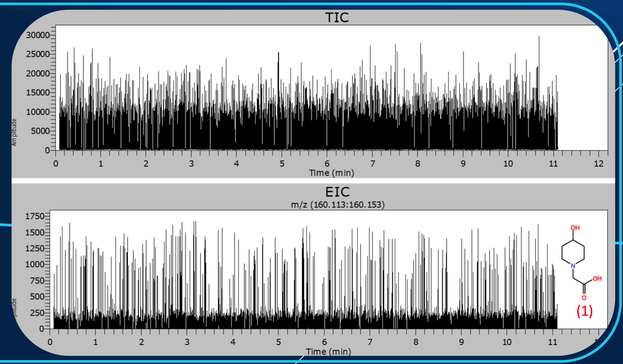
This plan view of a sorting biochip, shows the location of the optical fibre, close to the sorting junction. If the PMT voltage exceeds a set threshold an oscillating high voltage is applied to the electrodes. The use of the dielectrophoretic effect causes the picodroplet to move into the sorting junction.

ESI-MS Picodroplet Data

Typical picodroplet ESI MS data: shows a saw-tooth like appearance and also an expansion of five picodroplets, see below. Each data point represents one scan.



The two ion current chromatograms to the right show a mixture of two concentrations of (4-hydroxy-piperitine-1-yl)-acetic acid (1) in picodroplets at 1 mM and 150 µM. Eleven minutes of ESI MS picodroplet data is shown. The total Ion chromatogram (TIC, top) gives no clear indication of the presence of two different concentrations of compound (1) being present. However, looking at the extracted ion chromatogram for compound (1, EIC, bottom trace), their presence is clearly visible and would allow these picodroplets to be easily selected by selecting a suitable threshold intensity (*e.g.* >500) or much higher if the number of hits required was significantly less (*e.g.* > 1500).



Conclusions & Future Refinements

- Significant progress has been made in many aspects of this proof of concept project for High throughput ESI-MS screening of phenotypes that produce and release an enzyme into the supernatant of picodroplets.
- The adaption of a commercial HPLC MS TOF was successful, but did lead to significant microfluidic problems, notably high back pressure in the sibling B delay line. This will be fully resolved when the interface is reengineered.
- However, conducting the enzyme assay by adding the ketone substrate by either picodroplet fusion or picodroplet injection was not achieved in a way that allowed a set incubation period to be maintained prior to screening on the MS biochip. This will also be addressed during the building of an alpha-prototype.
- The use of pressure pumps for several aspects of this project should allow us to grow organisms that prefer other gas environments or even allow their use as isotopic mass labels, e.g. N^{15}.
- The software currently only currently operates at maximum scan speed of 30 Hz, which means that we are not maximising the full performance of the MS which can scan at 100 Hz. So further software optimisation is required..
- The light-scattering screening biochip has seen much improvement and can now operate at 300 Hz with 300 pL picodroplets. We hope to make this biochip available in 2017 (see: http://www.spherefluidics.com/store).
- This emerging platform, called ESI-Mine™, will allow the user to: 1) make highly monodisperse picodroplets, 2) Incubate under a select gas or mixture of gases, 3) Carry out light-scattering based sorting, 4) Allow enzyme reactions to be screened by ESI-MS, at a fixed time point, 5) The possibility to carry out on a biochip picodroplet fusion for scenarios where product modification or acidification is required to improve sensitivity.

References & Acknowledgements

1. C. Smith, X. Li, T. H. Mize, T. D. Sharpe, E. I. Graziani, C. Abell, W. T. S. Huck, "Sensitive, high throughput detection of proteins in individual, surfactant stabilized picolitre droplets using nanoESI mass spectrometry". *Anal Chem*, 2013, **85**, 3812-3816.
2. X. Liu, R.E. Painter, K. Enesa, D. Holmes, G. Whyte, C.G. Garlisi, F. Monsma, M. Rehak, F.F. Craig, C.A. Smith, "High-Throughput Screening of Antibiotic-Resistant Bacteria in Picodroplets", *Lab Chip*, 2016, **16**, 1636-1643.

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