High-throughput picodroplet-based analysis of biosynthetic libraries

Carlos Bricio[1], Xin Liu[2], Xin Li[2], Marian Rehak[2], Tom Ellis[3] and Clive Smith[2].

(1)Bioengineering, Imperial College London, London, United Kingdom, (2)Sphere Fluidics Limited, Cambridge, United Kingdom, (3)Centre for Synthetic Biology and Innovation, Imperial College London, London, United Kingdom

Abstract

Biosynthesis of high value chemicals by engineered microorganisms is an application of synthetic biology that offers both economic and environmental advantages. This application is increasing the need for high-throughput screening tools that can facilitate the detection of the best performance among a library of designed microbes. For this reason we are developing a high-throughput, miniaturised Mass Spectrometry (MS) tool for profiling synthetic designed libraries.

Combining microfluidics based picodroplet technology for cell encapsulation and sorting together with Mass Spectroscopy we aim to rapidly screen, identify and retrieve the best cell "hits" among synthetic metabolic pathway libraries. Based on this novel approach we will be able to determine which construct has the genetic combination that gives the best biosynthesis performance. To test this new tool we have designed three libraries of two synthetic metabolic pathways using molecular engineering techniques [1,2,3].

We chose two previously described synthetic pathways to produce non-natural amino acids [4, 5] and focus on improving their level of expression. Various strategies have been explored such as the use of homologue genes from other organisms, varying the DNA copy number, transcription levels or translation activity. Then, using a pioneering picodroplet-based technology [6] that enables not only the testing of up to 200,000 samples per day by MS, using miniaturised input volumes (400-700 pL), but also for retrieving identified 'hits' in a reproducible manner, we will select single cells, analyse their production of this non-natural amino acids and finally select and recover the best performing clones among the different profiles obtained for further studies.

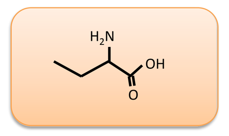
This will enable new scientific breakthroughs, higher throughputs, lower screening costs, shorten design-build-test cycle and thus, be of interest to the current MS user base in the synthetic biology market and other sectors.

DESIGN OF SYNTHETIC LIBRARIES

Metabolic pathway selection

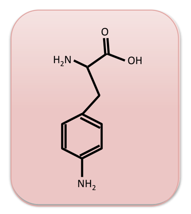
Two **non-natural amino acids** were chosen as target molecules for the screening:

L-Homoalanine

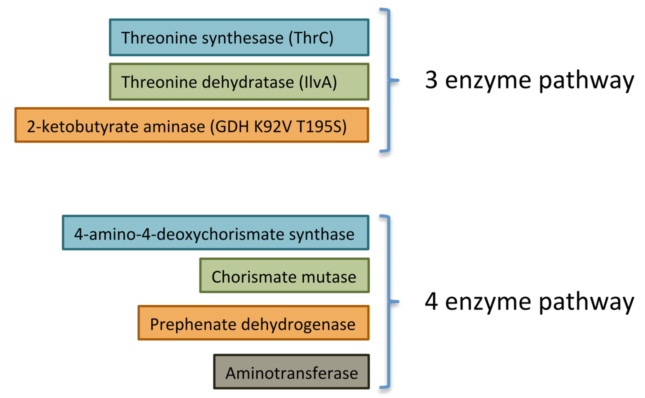


- Supplement to *in vitro* maturation medium for culture of oozytes and embryos.
- Chiral precursor of antiepileptic and antituberculosis drugs

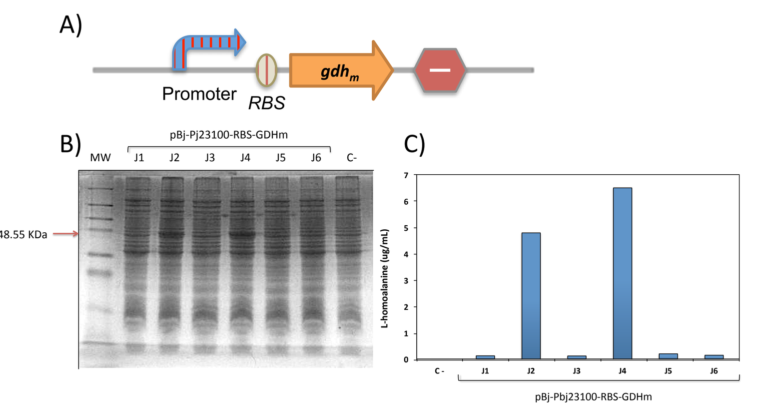
P-aminophenylalanine



- It is a secondary metabolite in the production of chloramphenicol and pristinamycin.



DIAL strain strategy



- **2-ketobutyrate aminase** gene (gdh_m) was cloned in pBj2992 plasmid.
  - The enzyme is responsible for catalyzing the synthesis of L-homoalanine.
- Use of 3 **DIAL strains** to vary the copy number of the plasmid.
- **Promoter library Pj23100**: 5'-ttKacRgctagctcagtcctaggtaYWRtgctagc-3'
- **RBS library** (RBS library calculator): 5'-tcccgcMtttataactaaggMGRtattc-3'

Figure 1: Preliminary analysis of the DIAL strain library producing L-homoalanine. **A)** 2-ketobutyrate aminase expression cassete designed in the pBj2992 vector. Red stripes represent the randomized sequence in the promoter Pj23100 (bended arrow) and the RBS (white circle). **B)** SDS-PAGE of the total protein extract from different clones from DIAL strain J expressing the gdh_m gene. MW lane shows the molecular weight marker and the red arrow indicates the 2-ketobutyrate expected molecular weight. **C)** Production of L-homoalanine by the same set of clones. C- shows the results from a strain without plasmid (negative control).

Golden Gate Assembly Strategy

- Using **MoClo Pro** kit (Modular Cloning)
  - Randomized RBS for each gene.
  - The restriction enzyme *Bpi*I is used for assembly the final construct.
- **12 promoters** including:
  - Constitutive promoters with different strengths. Including members of the Anderson Promoter Collection.
  - Growth inducible promoters, like PosmY.
- Bank of **31 genes** for the two pathways.
  - Including homologue genes for each enzyme from different organisms.

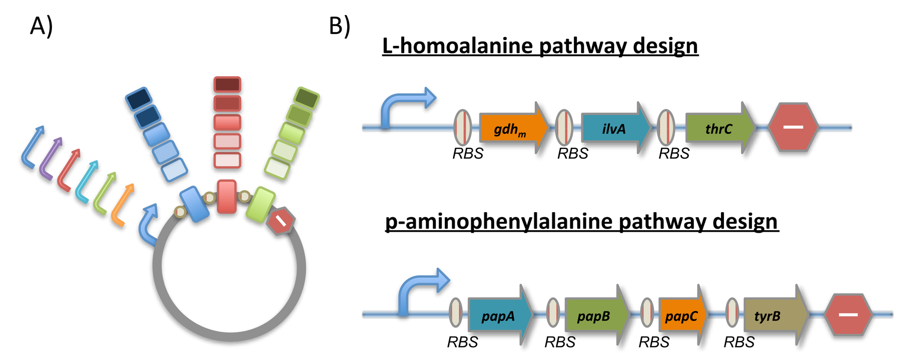


Figure 2: Plasmid design for the Golden Gate assembly strategy. **A)** Sketch of the plasmid construction using MoClo Pro. Different shades of colors represent different homologues of each ORF. Colored bended arrows represent different operon sequence. **B)** Structure of the two pathways operons to be assembled using the MoClo Pro kit. Red stripes represent the randomized sequence in the RBSs.

Dynamic Light Scattering (DLS) based Pre-sorting

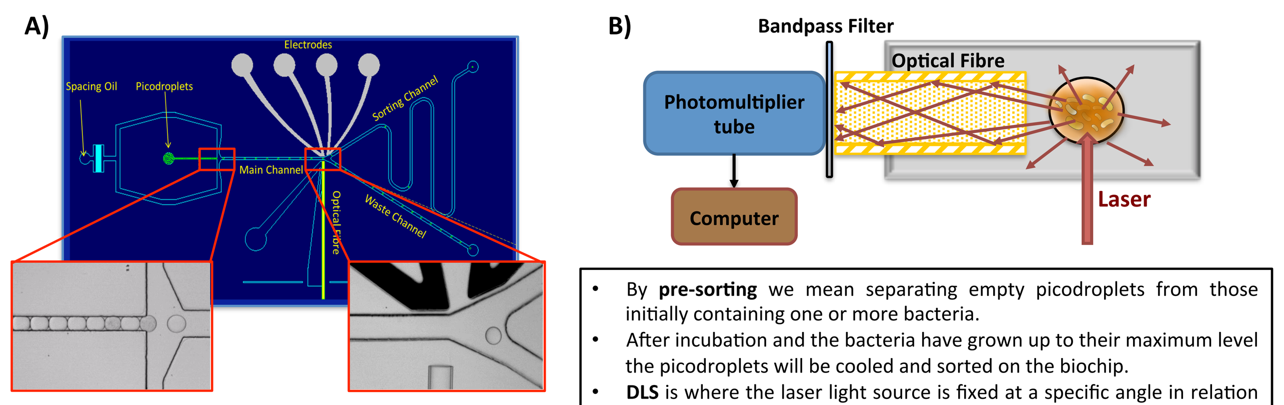
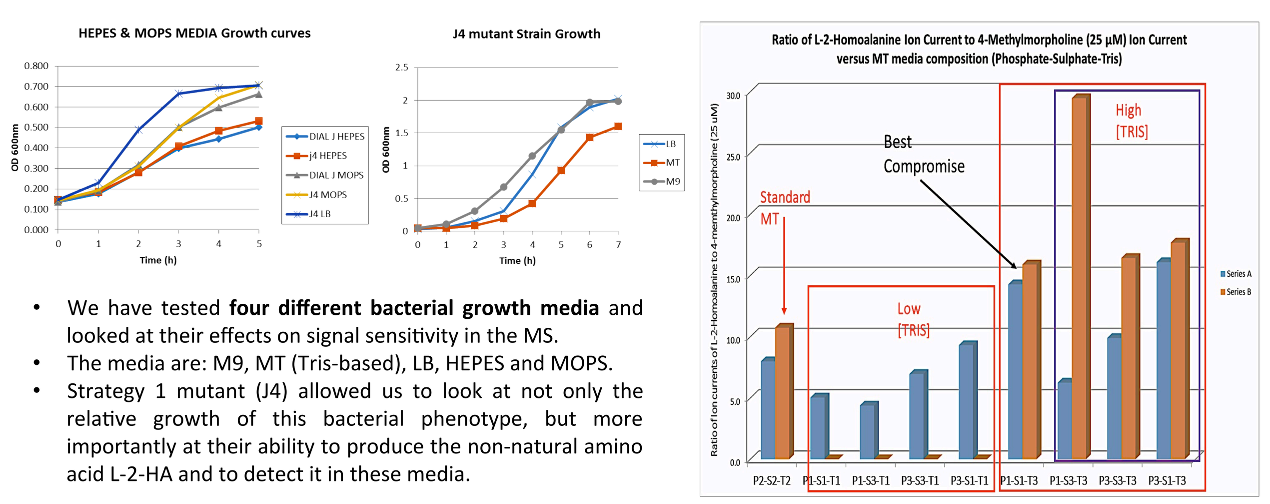


Figure 4: Pre-sorting picodroplets. **A)** Top view of the sorting biochip. The right close-ups shows the DLS sorting junction. **B)** Sketch of the side view of the Biochip.

- By **pre-sorting** we mean separating empty picodroplets from those initially containing one or more bacteria.
- After incubation and the bacteria have grown up to their maximum level the picodroplets will be cooled and sorted on the biochip.
- **DLS** is where the laser light source is fixed at a specific angle in relation to the optical detector (photopultiplier tube). Here the angle is 90˚.
- Light is scattered or reflected whenever there is a change in the refractive index on going from one medium to another.

Bacterial Growth Media Optimization



- We have tested **four different bacterial growth media** and looked at their effects on signal sensitivity in the MS.
- The media are: M9, MT (Tris-based), LB, HEPES and MOPS.
- Strategy 1 mutant (J4) allowed us to look at not only the relative growth of this bacterial phenotype, but more importantly at their ability to produce the non-natural amino acid L-2-HA and to detect it in these media.

Single Cell encapsulation in picodroplets

- **Step 1: Picodroplet Generation**

Once the culture medium has been optimized for growth and MS suitability, one can then determine the required amount of dilution of an overnight culture in order to obtain the desired starting bacterial cfu level. If this is correct, then the level of occupied droplets will match that predicted by the Poisson statistics.

- **Step 2: Bacterial Incubation in picodroplets**

Dilute overnight culture to OD. 0.28, then dilute a further 500-fold and encapsulate in 500 pL picodroplets. Grow the bacteria for 23 hours at 37° C, shaking at 220 rpm, in MS friendly media.

Poisson Statistics

In order to control the level of single bacterial occupancy of picodroplets, we used Poisson statistics. We have set the bacterial encapsulation volume at 500 pL ($2*10^6$/mL) to help microfluidics. The variable (λ) is the cell number (cfu/ml) to picodroplet (number/mL) ratio.

Equation

$$f(k;\lambda) = \frac{\lambda^k e^{-\lambda}}{k!}$$

(Desired percentage level of single occupancy: κ = 1)

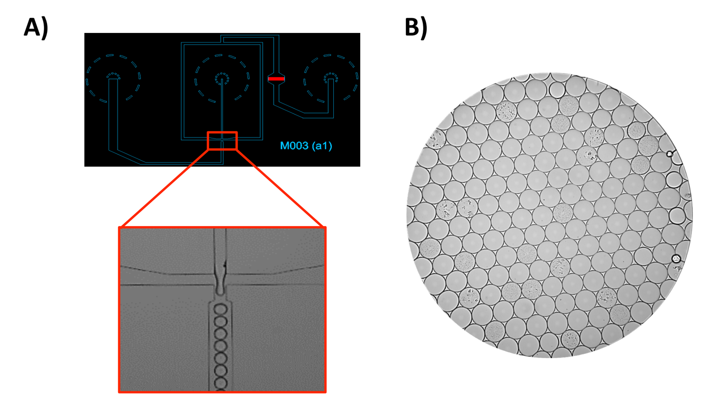
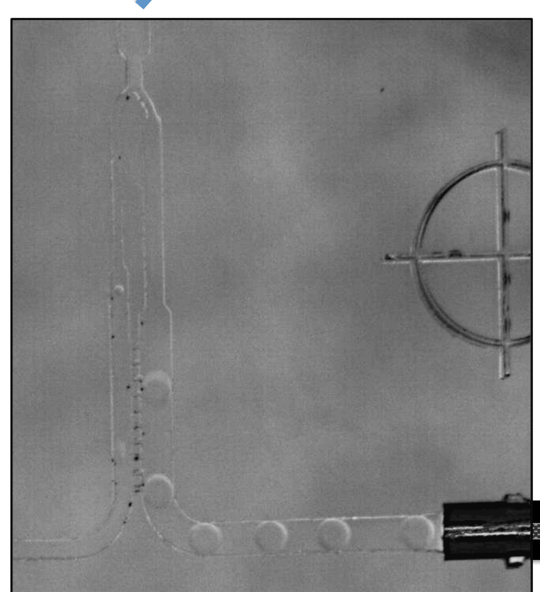
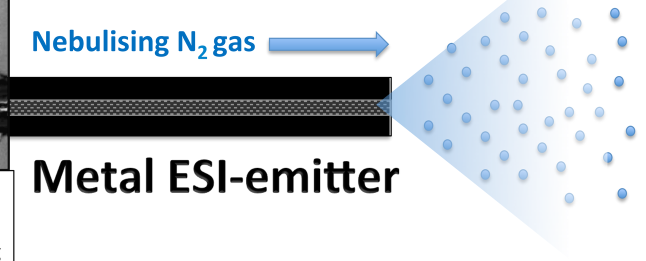


Figure 3: Encapsulation of single cells in picodroplets. **A)** Encapsulation biochip and close-up of the encapsulation junction. **B)** Picture of picodroplets showing approximately 12.5% encapsulation.





Picodroplet MS Biochip

Picodroplet MS biochip (micrograph) showing picodroplets being split with the larger sibling going in to the metal MS emitter. The smaller sibling picodroplet is retained on the biochip and can be selected and dispensed into a microtitre plate, for subsequent DNA analysis, should its larger sibling, that is being analysed by the MS, proves to be interesting.

PerkinElmer Axion 2 ESI MS

MS screening

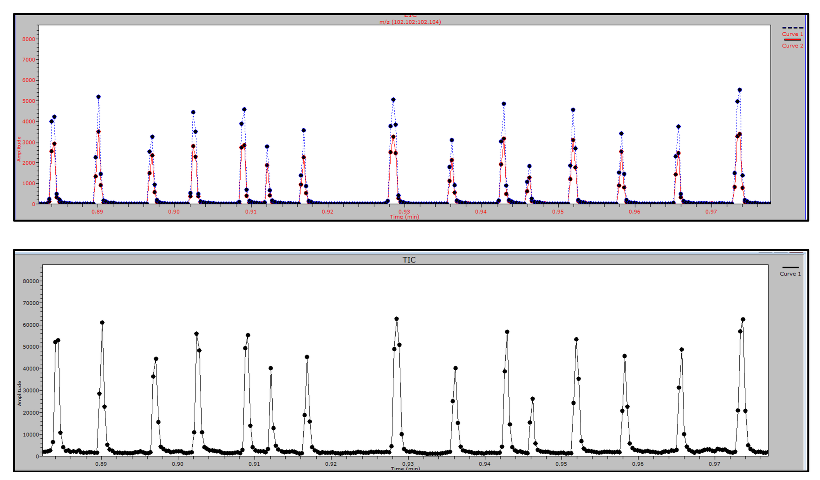
- **MS Library Size**

We are using a PerkinElmer Axion 2 ESI mass spectrometer. One of the reasons for choosing this instrument is not only its high scan rate (100 Hz), but also its relatively high mass resolution (12000 FWMH at m/z 922), in order to be able to visualize individual picodroplets in the TIC. Ideally would like to have at least 10 scans to characterize each picodroplet, this leads to a MS screening throughput of up to 10 Hz (36,000 picodroplets/ h).

Modelling ESI MS Throughput

MS Screening Frequency (Hz)	10	5
Screening Time (Hours)	6	6
Library Size	216000	108000
Picodroplet #	5363457	2681729
Picodroplet generation (Hz)	1600	1600
Picodroplet Generation Time (Hours)	0.99	0.50
Picoroplet Diameter (µm)	98.5	98.5

Data analysis



- 50 Hz Pulse mode ESI- MS of 500 pL picodroplets, showing the typical saw-tooth pattern in the TIC spectrum (above right). Below the TIC spectrum is an EIC spectrum showing the individual ion currents for L-2-homoalanine (blue trace) and a molecule used as an internal standard (m/z 102, red trace).
- Each dot represents a single scan and in this experiment there are 50 scans per second allowing individual picodroplets to be analysed.

Conclusions & Future perspective

- It has been previously shown that microfluidic picodroplets can be used as reactors to study single bacterial proliferation [7, 8]. The use of microfluidics in bacterial growth allow us to reduce the compartmentalization volume of bacterial cultures down to 500 pL. We have observed that picodroplet-based bacterial cultures do grow to a higher density than in 3 mL shake flasks. This technique will help to save screening costs and increase the number of samples that can be screened in a short period of time.
- We are currently increasing the variability of the synthetic libraries, optimising the microfluidics, growth media and developing the optics, barcoding strategy and software to analyse individual bacterial phenotypes that are producing non-natural amino acid.
- Ultimately, the recovery and analysis of the best hits after screening will allow to understand what combination of elements used in the synthetic library design is best for production and will help in further rounds of optimization.

References

1. Kittleson, J. T., Cheung, S., & Anderson, J. C. (2011). Rapid optimization of gene dosage in E. coli using DIAL strains. J Biol Eng, 5(1), 10.
2. Engler, C., Gruetzner, R., Kandzia, R., & Marillonnet, S. (2009). Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. PloS One, 4(5), e5553.
3. Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison, C. a, & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature Methods, 6(5), 343–5.
4. Mehl, R. a, Anderson, J. C., Santoro, S. W., Wang, L., Martin, A. B., King, D. S., *et al.* (2003). Generation of a bacterium with a 21 amino acid genetic code. J. Am. Chem. Soc., 125(4), 935–9.
5. Zhang, K., Li, H., Cho, K. M., & Liao, J. C. (2010). Expanding metabolism for total biosynthesis of the non-natural amino acid L-homoalanine. Proceedings of the Proc Natl Acad Sci USA, 107(14), 6234–9.
6. CA Smith, X Li, TH Mize, TD Sharpe, EI Graziani, C Abell, WT Huck (2013). Sensitive, High Throughput Detection of Proteins in Individual, Surfactant-Stabilized Picoliter Droplets Using Nanoelectrospray Ionization Mass Spectrometry. Anal. Chem. (85), 3812-3816.
7. Martin, K., Henkel, T., Baier, V., Grodrian, A., Schön, T., Roth, M., *et al.* (2003) Generation of larger numbers of separated microbial populations by cultivation in segmented-flow microdevices. *Lab Chip* **3**: 202–7
8. Huebner, A., Srisa-Art, M., Holt, D., Abell, C., Hollfelder, F., deMello, A.J., and Edel, J.B. (2007) Quantitative detection of protein expression in single cells using droplet microfluidics. Chem Commun (Camb) 1218–20.

Funding: This project between Imperial College London and Sphere Fluidics Limited is jointly supported by a Technology Strategy Board Grant and the BBSRC.