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Small or medium-scale focused research project (STREP)

Deliverable 1.1 - Report about full characterization of BZ reaction in lipid-enclosed droplets

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Droplet-to-Droplet Communication in the Lipid-Enclosed Belousov-Zhabotinsky Reaction**

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The Belousov-Zhabotinsky (BZ) oscillating reaction involves the bromination and subsequent catalysed oxidation of an organic substrate.[1, 2] The BZ reaction has been previously used in water-in-oil microemulsions, stabilised by the detergent aerosol-OT (AOT).[3] The AOT microemulsions comprise inverse detergent micelles with an internal aqueous diameter of ~1 nm (μL volume) and give rise to Turing patterns, generated by the diffusion of reactive species through both the compartmentalized aqueous phase and the bulk oil phase. Pattern formation has also been observed when the BZ medium is supplemented with phospholipids that self-assemble in nanometer-thick bilayer sheets,[4, 5] although the size and interconnection of the lipid-delimited aqueous compartments is not known. In a microtechnology-inspired approach, Epstein and co-workers and Seemann and co-workers have very recently employed microfluidics to generate water-in-oil 'macroemulsions' in which the aqueous phase of BZ medium is present as a 1D or 2D array of ~100-200 μm diameter droplets that can be imaged individually.[6-8] It was observed that synchronization of BZ oscillations, which implies diffusion of certain BZ components from droplet to droplet, depends on the droplet spacing and the presence of surfactants in the oil phase.

Here we employ a fabrication strategy based on 3D printing to position droplets of two different BZ mixtures at discrete locations in an oil reservoir. This flexible new approach to compartmentalise BZ reactions highlights the requirement to effectively maintain the BZ reaction conditions within the droplets and identifies conditions that enable droplet-droplet communication. Microdroplets are increasingly explored for a wide range of applications, including the miniaturization of (bio)chemical reactions, the study of reaction kinetics, the manipulation of single cells, and the formation of interdroplet lipid bilayers for ion channel electrophysiology.[9-11] Typically, detergent molecules are added to the oil phase to stabilize the droplets by the formation of a surfactant monolayer at the oil-water interface. While large biomolecules will be effectively contained within the aqueous phase, small molecules with a hydrophobic character could diffuse into the oil phase.[12-14] Perfluorinated oils have a reduced solubility for non-fluorinated compounds and are therefore routinely used to suppress droplet leakage and avoid droplet cross-talk.[15] In the case of aqueous droplets of BZ medium, separated by oil slugs, in microchannels, synchronization of BZ oscillations over the linear droplet array is only observed in the absence of unsaturated surfactant and when the length of the oil slug is comparable to the diameter of the droplet.[6-8] Interestingly, it was very recently reported that an excitation wave can travel through a planar droplet array when detergent-coated droplets are in direct contact.[7]

A number of variations of the BZ reaction exist, with a variety of substrates and catalysts. Iron-based catalysts, such as $[Fe(o\text{-phen})_3]SO_4$ (ferroin) are frequently used to provide better visual contrast,[16] while the substitution of 1,4-cyclohexadione (CHD) is used to overcome the carbon dioxide evolved during the reaction with malonic acid (MA).[17, 18] Here we investigated oscillations of

a gasless, self-excitable BZ ferroin mixture as droplets in a reservoir of decane oil in the presence and absence of the lipid mixture asolectin as surfactant (20 mg ml^{-1}).

Droplets were positioned using custom-designed wells with 1 mm wide by 0.5 mm deep trenches, adapted from the polydimethylsiloxane (PDMS) 'droplets on rails' devices published previously.[19] Previous studies on the CHD-BZ reaction in microfluidic devices have shown that the BZ reaction is incompatible with PDMS, likely caused by transport of bromine from the aqueous phase into the PDMS elastomer;[20] we observed no oscillations in PDMS devices. The use of decane as the organic phase is also problematic as PDMS is solubilised by decane, causing swelling of the channels. We overcame these issues by fabricating wells from Norland optical adhesive No. 81 (NOA-81) doped with 1% 3-aminopropyltriethoxysilane (APTES) to increase the hydrophobicity.[21] The rapid fabrication of the wells by 3D printing the desired device geometry, and creating a PDMS mould, followed by casting the device in the NOA-81/APTES mixture allowed several droplet geometries and spacings to be explored (see supplemental information). Droplet arrays were created from droplets of 2.5 ± 0.2 µl volume, unless otherwise stated. It should be noted that, unlike previous studies[6-8] the droplets were covered by a bulk oil phase instead of being confined in a microchannel separated by small oil slugs.

For isolated droplets of the CHD mixture in decane or in the fluorinated oil FC-40, no oscillations were observed; the CHD-BZ mixture remains red without oscillation for time periods greater than four hours. In contrast, the MA-BZ mixture did oscillate when immersed in a pure oil phase. These oscillations are characterised by a wave of blue, oxidised ferroin travelling across the previously red droplet. In the presence of lipid in the decane oscillations occured for both BZ mixtures. These observations suggest that one or more of the reactive species in the CHD-BZ mixture leak away into the oil phase, but are retained in the aqueous droplet when it is covered by a monolayer of asolectin lipids. As the MA system is unaffected by the absence of lipid, this reactive species is likely to be the bromo-cyclohexadione intermediate.

Next, the oscillatory behaviour of BZ in lipid-enclosed droplets was assessed for the CHD and MA mixtures in more detail (see figure 1). Droplets of volumes between 0.5 µl and 4.5 µl were screened for their oscillatory properties, and those of 2 µl volume or greater were found to display oscillations in the presence of asolectin/decane (20 mg/ml). This threshold volume for droplet reactions can be accounted for by the oxygen inhibition observed previously in droplets of MA-BZ.[22] The mean oscillation period was around 7 minutes for droplets of CHD-BZ, while oscillations in droplets containing MA-BZ were much quicker, at around 2 minutes. Droplets containing CHD-BZ showed oscillations that typically started at one side of the droplet, spreading across the whole droplet, whereas in the MA system, oscillation waves started in the centre of the droplet and spread outwards (see supplemental information). For both reaction mixtures, droplets of volume > 3 µl showed constant oscillation periods; droplets of smaller volume show fewer oscillations, at periods that differ slightly from the bulk phase. This threshold droplet volume is in agreement with droplet size requirements observed in similar studies.[23]

We created arrays of droplets with well-defined interdroplet spacings ranging from 100 to 250 µm, which is the limit of our fabrication method. It was found that at small droplet-droplet distances (≥ 0.25 mm) the BZ reactions in the droplets are independent, but when the droplets are in contact, the BZ oscillations are not independent, and communication is observed across the lipid bilayer. Communication was observed between the asolectin-enclosed droplets, starting from a single point determined by the system and spreading until another wave of excitation was encountered (figure 2 and videos in supporting information). It should be noted that in the absence of lipids the droplets cannot be brought into close proximity without merging.

Arrays of lipid-enclosed droplets represent a well-documented method for forming droplet interface bilayers (DIBs). The stability of the bilayer separating individual droplets was found to be linked to the concentration of reagents in the BZ mixture, in particular the concentrations of H_2SO_4 and BrO_3^-. An increase in the concentrations of either species results in destabilisation of the bilayer and merging of the droplets.

It is hypothesised that the communication observed between the droplets results from acid-catalysed hydrolysis of the lipid species, and subsequent reorganisation of the lipid bilayer, analogous to the uptake of cationic amphiphilic drug (CAD) molecules[24, 25] or by diffusion over the bilayer.[26] Hydrolysis of dioleoylphosphatidyl choline (DOPC) membranes by CADs yields lyso-PC and oleic acid (OA),[24] resulting in a mixture that forms an inverse hexagonal phase.[27, 28] The asolectin used in this study contains a mixture of phospholipid species, including a significant amount of phosphatidyl ethanolamine (PE, 18%), which has an intrinsic preference for negative curvature,[29] and would therefore facilitate destabilisation of a bilayer in this manner.

Hydrolysis was simulated in our system by using mixtures of asolectin doped with OA (up to 20 wt%). It was observed that at higher OA concentrations the BZ mixture in the droplets began oscillating earlier than experiments in which no OA was added, and that the relative stability of the droplets decreased with increasing OA concentration. At 20 wt% OA, three droplets had merged into a single droplet within 10 minutes of the introduction of BZ droplets to the oil system (supporting information). It was also found that merging was more likely when the excitation wave front reached the membrane separating individual droplets, behaviour identical to that observed with increasing acid concentration. This droplet merging, and an associated fluctuation in apparent droplet shape, is likely linked to the change in interfacial tension observed with the passing of BZ waves in water in oil droplets.[30]

In summary, this study details the characterisation of an oscillating reaction medium in lipid-enclosed droplets in decane, that for the CHD-BZ system the presence of a lipid envelope is essential for oscillations in droplets. For experiments containing two or more droplets of CHD-BZ arranged in a linear array, communication of the excitation wave was observed to pass across droplets separated by the asolectin/decane phase. Excitation starts from a point (or points) in the droplet array, usually located at the oil-water interface of a droplet and spreads outwards from this point.

Our approach builds on previous attempts to compartmentalise the BZ reaction[6-8] and allows individual droplets of aqueous BZ medium to be brought together in an excess of organic phase without merging. The lipid envelope surrounding our droplets acts as a barrier between compartments but allows transfer of chemical species between compartments that are in contact. It is our aim to build on these observations to create a system of compartmentalised BZ droplets for processing information.

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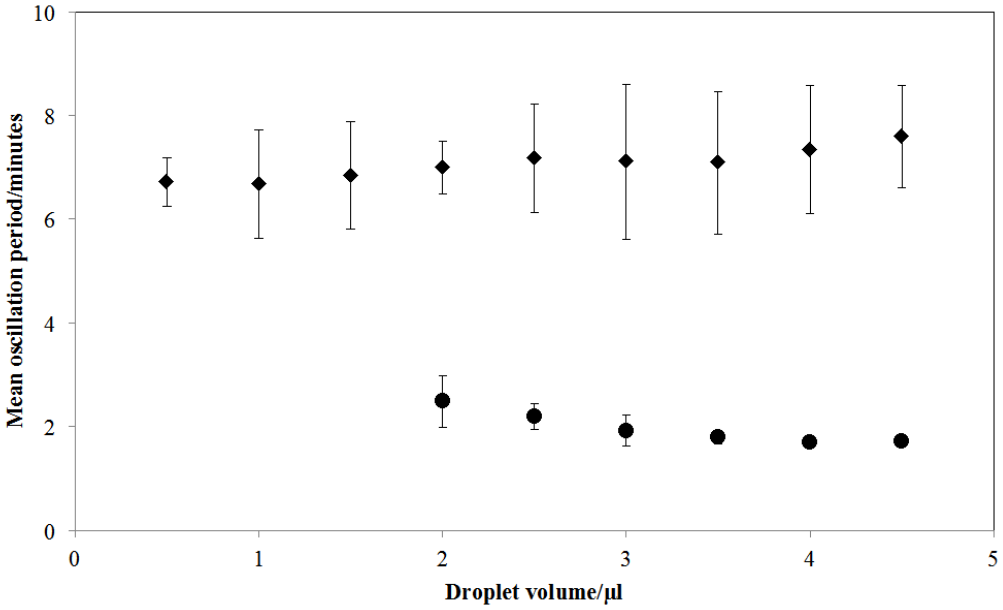


Figure 1. Oscillatory periods of lipid-enclosed BZ reactions plotted against droplet volume. Oscillatory periods are shown for droplets containing the CHD reaction mixture (diamonds) and the MA reaction mixture (circles) with reaction concentrations: H_2SO_4 200 mM, $NaBrO_3$ 350 mM, substrate 175 mM, and ferroin 1.7 mM. Droplets were enclosed by asolectin/decane, 20 mg/ml. Error bars represent the standard deviation of the oscillation periods of ten sets of droplets.

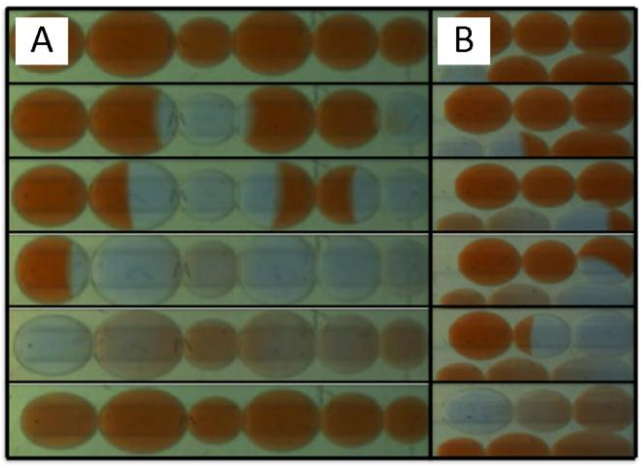


Figure 2. The wave of oxidation of the ferroin catalyst is spread between droplets of asolectin enclosed BZ. A: The wave travels across the row of droplets, beginning from the third droplet in the row, and progressing outwards. Another wave travelling from the right hand side of the image meets the first and annihilates. B (from top): the bottom left hand droplet of the array passes a wave of oscillation towards the right, this excitation is passed upwards on the right hand side of the array, and the wave then travels back round to the left. See supplemental information for videos.

Supporting information for

Characterisation of the ferroin-catalysed Belousov-Zhabotinsky reaction in lipid-enclosed droplets

Josephine C. Corsi,[a] Philip H. King,[a] Hywel Morgan,[a] Maurits R. R. dePlanque[a] and Klaus-Peter Zauner*[a]

1) Experimental details

1.1) Preparation of BZ reaction mixtures

Belousov-Zhabotinsky reaction mixtures were prepared with the following concentrations: 200 mM H_2SO_4, 350 mM $NaBrO_3$, 175 mM substrate (either 1,4-cyclohexanedione or malonic acid), 1.7 mM ferroin. Reaction mixtures were incubated at room temperature until oscillations of reaction colour began, in the case of the malonic acid this was < 5 minutes, for cyclohexanedione incubation took 4 hours. Droplets of reaction mixture were then introduced to the organic phase for experiments. All chemicals were purchased from Sigma Aldrich.

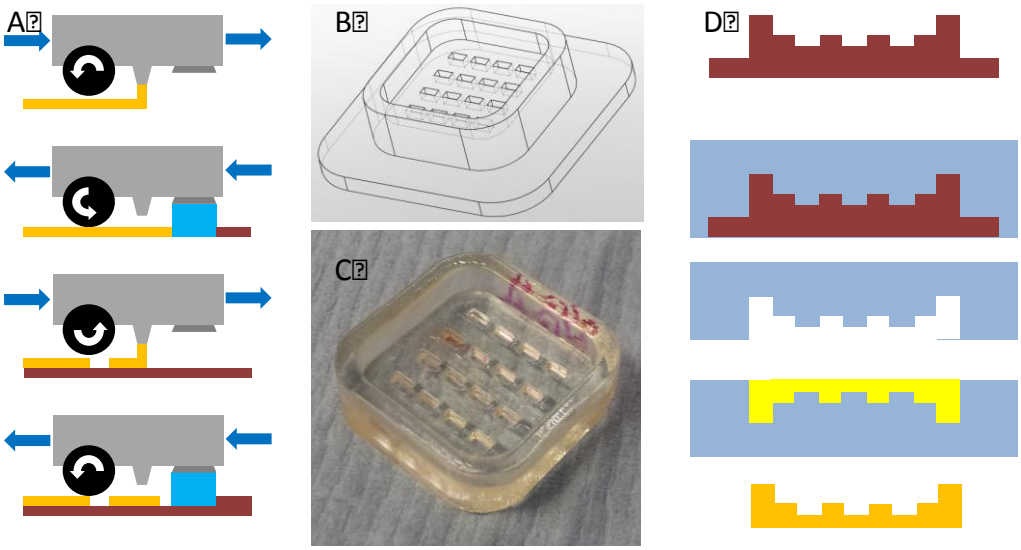
1.2) Preparation of lipid/decane mixtures

Lipids were purchased as lyophilized powders (Avanti Polar Lipids, Alabama, USA) and desired masses were weighed out. Lipids were then dissolved in sufficient decane (Sigma Aldrich, UK) to produce required concentrations (20 mg/ml unless stated) and sonicated to ensure complete dissolution.

1.3) Fabrication of NOA-81 droplet wells

A 3D CAD model of the droplet positioning device was produced using SolidWorks 2009 (Dassault Systemes) and exported in .stl format. The mould was printed using a Connex 350 3D printer (Objet Geometries Ltd.), with FullCure720 (pictured) or VeroWhite as the bulk material and FullCure705 as the support. The support material was removed using a water jet. The mould was baked overnight at 80°C to prevent an inhibitory effect on the curing of PDMS. It was then silanized using trichloro(1H,1H,2H,2H-perfluorooctyl) silane (Sigma-Aldrich) by exposure to vapour under partial pressure in a desiccator.

PDMS (Sylgard 184 Elastomer, Dow Corning) was mixed at 10:1 monomer to curing agent and degassed. The mixture was poured into a foil boat containing the mould, degassed again and baked at 80°C for 1 hour to cure. The demoulded cured PDMS was used as a mould for NOA81 UV-curable adhesive (Norland Products) with 1% (v/w) 3-amoinopropyltriethoxysilane (APTES, Sigma-Aldrich), which was cured using a 100W Blak-Ray B100AP High Intensity 365 nm UV lamp (UVP) for 60 minutes. The cured NOA81 was then demoulded ready for use.



1.4) Droplet experiments

Devices were filled with the asolectin/decane mixture prior to the introduction of droplets, of typical volume 2.5 µl. Video was captured using a Zeiss Stereozoom microscope coupled to a Prosilica GC2450C digital camera (Allied Vision Technologies) camera. Video was recorded at a frame rate of 30 frames per second, with 28 frames being discarded for every frame captured.

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Attachement to Deliverable 1.1 - Status of Deliverable D1.1 after year 1

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Start date of project: 1.2.2010	Duration: 36 months
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Status of Deliverable 1.1

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Project acronym: NEUNEU
Project title: Artificial Wet Neuronal Networks from Compartmentalised Excitable Chemical Media
Document Version: 14/03/2011

Work package 1.1 – Belousov-Zhabotinsky oscillation in a single lipid-enclosed droplet

T1.1 Screening of BZ Mixtures

Early research into was carried out on large random populations of super-excitory droplets in a continuous phase of decane containing asolectin (60 mg/ml). The droplets were formed by the manual agitation of ferroin-catalysed malonic acid (MA)-substrate BZ media, mixed in vials with the decane/lipid phase, with the resultant mixture poured into a petri dish to give a single layer of droplets

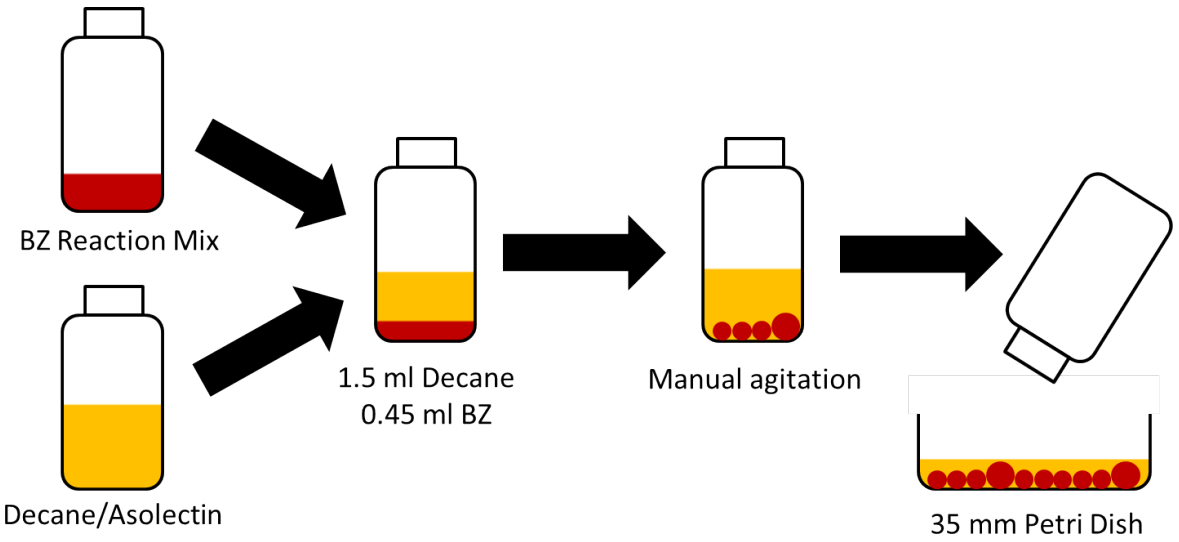


Figure 1: *Manual production of droplets of ferroin-catalysed BZ media via agitation.*

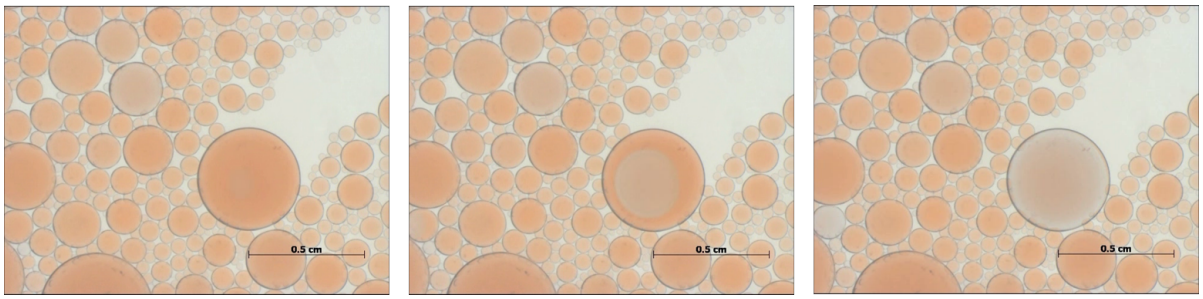


Figure 2: *Self-excitation of large ferroin-catalysed BZ droplet in decane/asolectin mixture. Droplets created using method outlined in* Figure 1*. [Acid] in BZ = 330 mM. Scale bar represents 5 mm.*

A number of tests were carried out in this fashion, varying the final sulphuric acid concentration between 0.1 and 0.45 M as per previous experiments by Szymanski *et al* (Warsaw). The droplets were found to self-activate after an incubation period of around 5 minutes and then oscillate for 30-60 minutes afterwards.

Although super-excitory media remains an interesting test bed for BZ reactions in droplets, it is possible that their independent internal oscillation mechanics makes them unsuitable when looking for droplet-to-droplet interactions. Excitory BZ media was prepared following the method in Showalter & Noyes[1] where the self-excitability of the media is controlled by controlling the ratio of final concentrations of sulphuric acid to sodium bromate. If $[H_2SO_4] \times [NaBrO_3] \leq 0.045$ M^2, the mixture will not self-excite, but is excitable by exposing the mixture to silver wire. This leads to the production of silver bromide, which precipitates, reducing the localised bromide concentration below the excitability threshold, thus initialising an excitation wave.

Development of the chemistry from this point onwards was carried out by Josephine Corsi

The evolution of carbon dioxide from traditional BZ reactions using malonic acid as the substrate was discovered to be particularly problematic for droplet-based and microfluidic systems, and so a gasless version of the BZ reaction was developed. The traditional substrate of malonic acid (MA) was substituted with a variety of compounds including acetyl acetonate, and 1,4-cyclohexadione (CHD). Versions of an oscillating BZ reaction using CHD have been reported in the literature,[2-4] although at significantly high concentrations of sulfuric acid (2 M), which were observed to result in the merging of separate lipid-enclosed droplets, presumably through hydrolysis of the lipid monolayer. A range of concentrations under which clear oscillations were observed inside lipid-enclosed droplets, without perturbation of the monolayer was mapped. Multiple droplets were used to assess the stability of individual droplets; reaction conditions that caused neighbouring droplets to merge were deemed unsuitable for this project.

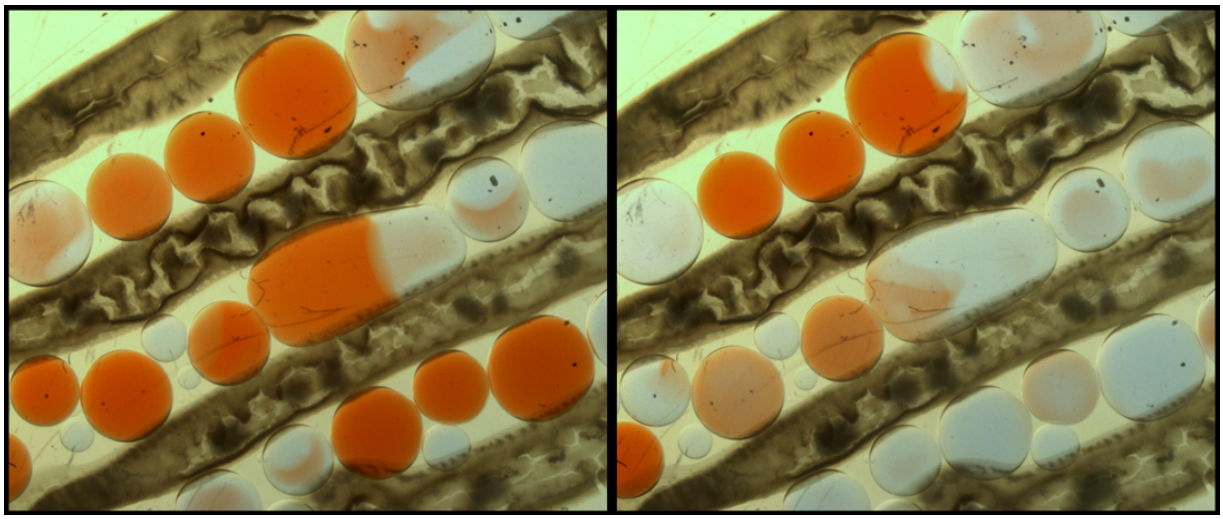


Figure 3: *Droplets of CHD BZ (300 mM H2SO4, 450 mM NaBrO3, 350 mM CHD, 1.7 mM Ferroin) in asolectin/decane (60 mg/ml), separated by ridges of super glue approximately 1mm wide in a petri dish.*

Chemical	[Upper limit]/mM	[Lower limit]/mM	[Preferred]/mM
Sulfuric acid	300	100	200
Sodium bromate	400	300	350
1,4-cyclohexadione	>600	150	175
Ferroin	34	34	34

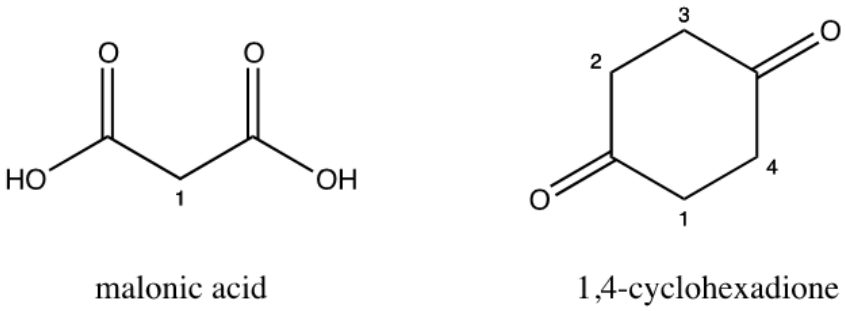
Table 1: *Concentration limits of reagents used to produce BZ reactions suitable for oscillations in lipid-enclosed droplets.*

This particular version of the BZ reaction does not appear in the literature (to the best of the author's knowledge), although is derived from methods developed by other groups.[2-4] The maximum concentration of CHD explored was 614 mM, oscillations were still observed at this concentration although greater concentrations could not be made under the constraints of this experiment.

T1.2 Characterisation of selected BZ mixture

The CHD version of the BZ reaction exhibits reaction kinetics that are significantly different to those of the MA reaction. The most striking difference is that there is a long incubation period that derives from the enolisation rate of CHD in comparison with MA. The initiation of the reaction involves bromination of the substrate at the α-carbon position (defined as the carbon atom immediately adjacent to a functional group, in this case the carbon bound to the carbonyl). In malonic acid this reaction occurs relatively quickly (indeed, some versions of the reaction include the inhibitory potassium bromide to delay this step), although in malonic acid the α-carbon is much less reactive. Once the incubation period has passed (between three and four

hours during which the ferroin in the reaction remains in the blue, oxidized state) oscillations occur steadily at intervals of a minute or so for at least two hours. The oscillations observed from the CHD mixture are much more vividly coloured, and easier to see than those of the malonic acid reaction. The oxidation front of the reaction (the blue wave) is much broader than is observed in the malonic acid system, making the CHD system excellent for viewing droplet-based reactions using light microscopy.



malonic acid 1,4-cyclohexadione

Figure 4: *Malonic acid contains one alpha carbon, labeled position 1. 1,4-CHD contains four alpha carbons.*

It was observed that there is an optimum droplet size for droplet based BZ reactions using CHD. Droplets that are too small (typically less than 1 µl) do not exhibit many oscillations before returning to the oxidized state, while droplets that are too large (greater than 5 µl in the current experimental setup) appear to merge easily with neighbouring droplets. Droplets of moderate size may exhibit a few oscillations (a maximum of 5) before becoming totally oxidized. Perhaps somewhat counter-intuitively, raising the concentration of CHD appears to cause a reduction in the number of oscillations rather than an increase, with the optimum concentration of CHD currently estimated at around 175 mM.

The oscillations observed are greatly affected by the choice of surfactant used in the system. The original experiments were carried out in asolectin (lecithin from soy beans, which contains a mixture of phospholipids), at the relatively high concentration of 60 mg ml^{-1}. Other surfactants, such as sorbitane monoleate (Span 80), octylphenol ethoxylate (Triton X-15), polyoxyethylene (2) oleoyl ether (Brij 93), have been used to explore their ability to stabilize BZ-containing droplets. It was observed that in the presence of a high concentration of Span 80 (50 % in decane) the reaction was noticeably slower than in asolectin. Other surfactants have also been shown to disrupt the BZ reaction.[5]

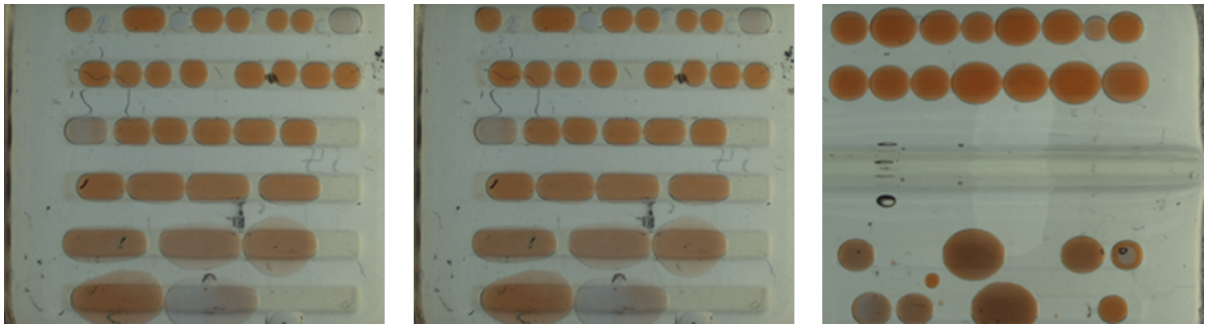


Figure 5*: Droplets of CHD BZ under different surfactant conditions. Left: Asolectin/decane (60 mg/ml), droplets with volume between 0.35 µl and 4 µl. Middle (top; bottom): Span 80/ decane (1:1 w/w); Brij 93/decane (1:1 w/w). Right (top; bottom): Span 80/decane (1:1 w/w); Triton X-15/decane (1:2 w/w). The rails holding the droplets have a width of 1 mm.*

The choice of surfactant is greatly influenced by the chemistry of the BZ reaction. Some of the surfactants used (most notably Triton X-15, but to some extent Span 80) caused a high degree of droplet merging, so that assessing the effect of droplet size in these systems was not possible. Constraints on the surfactant chemistry of the system are mostly linked to the ability of the surfactant to stabilize water in oil emulsions, but also how susceptible the surfactant is to hydrolysis by the BZ medium. Standard phospholipids are notoriously labile to hydrolysis at the ester linkage between the head and tail groups. One solution to this is to select lipids that are of bacterial origin and are linked by an ether group rather than an ester group, although these can be quite costly. The expense may be reduced by incorporation of the lipid into the aqueous phase of the reaction rather than the organic phase, in the form of vesicles. Aqueous droplets containing vesicles have been shown to produce droplets encased by a monolayer of lipid in a method that is routinely used to create asymmetric droplet induced bilayers for studying membrane proteins.6 Exploration into the surfactant chemistry of the system will be ongoing.

T1.3 Establishing excitation conditions for BZ mixture

Investigations were made into triggering oscillation in the malonic acid BZ reaction. Excitation was induced inside droplets by insertion of agar-coated silver wire, which shows promise for the possibility of using electrochemical techniques to control the reaction in individual droplets. Droplets were also easy to move around the reaction chamber using the wire. The reaction was also triggered in individual droplets by means of using a pipette tip modified to contain a piece of sliver wire. This was used to ensure that droplets containing the malonic acid version of the BZ medium were inserted into the oil phase in their oxidized (blue) state, thus ensuring that all droplets had a uniform starting point. Attempts were made to use droplets containing oscillating super-excitory BZ medium to induce oscillation in neighbouring droplets containing and excitory reaction mixture were unsuccessful.

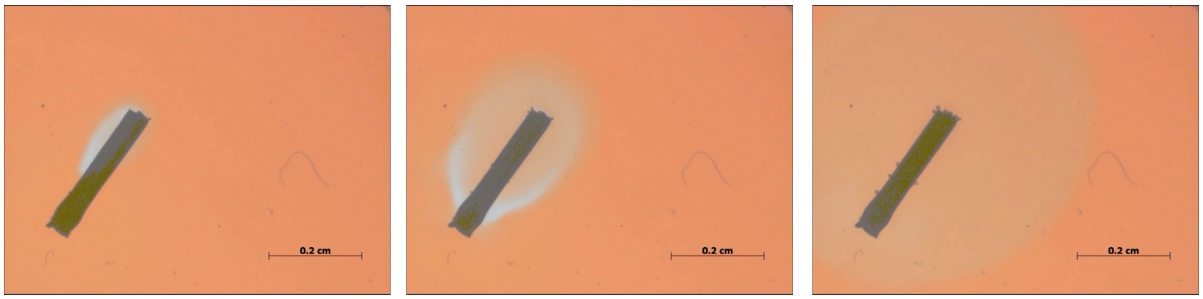


Figure 3: *Trigger of the excitory BZ reaction using malonic acid as a substrate, by silver wire, image provided by Philip King.*

So far the CHD reaction has not been used to set up an excitory mixture; all the CHD mixtures explored have been super-excitory. When reducing reagent concentrations in this system, the usual observation is an increase in incubation time with the solution remaining in the blue oxidized state. This means that initiation of a reaction using silver wire is not suitable, as the system is already oxidized. It has been observed, however, that solutions that are not yet exhibiting oscillation, but are within 30 minutes of their expected start time may begin oscillations shortly after being shaken. This observation, coupled with the observation that the CHD BZ reaction often exhibits a change in oxidation state when being drawn into a syringe, suggests that the reaction may be induced by a force acting on the system.

Controlling the CHD reaction using light appears to be more complex than the malonic acid reaction.7 Literature reports suggest that contrary to the malonic acid reaction that is inhibited by light of wavelength 450 nm, the cyclohexadione reaction is not inhibited under these conditions, and may even be stimulated. Electrochemical methods[1,8] have not yet been explored, although utilizing standard electrochemical techniques should yield information on the chemical nature of the reaction itself, as well as the possibility of learning new information about reactive intermediates in this system. There are plans to explore this further.

Microfluidic production of BZ droplets:

The field of microdroplet production has seen a large expansion in recent years, riding upon the rise of micro- and nano-fluidic chips towards lab-on-a-chip applications.[9] For the purposes of this project, the use of microfluidic techniques will allow the automated production of droplets of precise volumes on demand, the positioning and handling of said droplets, and in future the production of droplets of varying composition.

There are a number of now standard methods for the production of droplets in microfluidic systems. The simplest is the “cross-flowing steams” method, where the aqueous sample of interest is flowed into a perpendicularly-flowing column of the continuous (oil) phase. Another method is the “flow focussing” technique, where the aqueous stream is pinched into droplets by a pair of converging continuous phase flows. Both these concepts are outlined in *Figures 4* and *5* respectively.

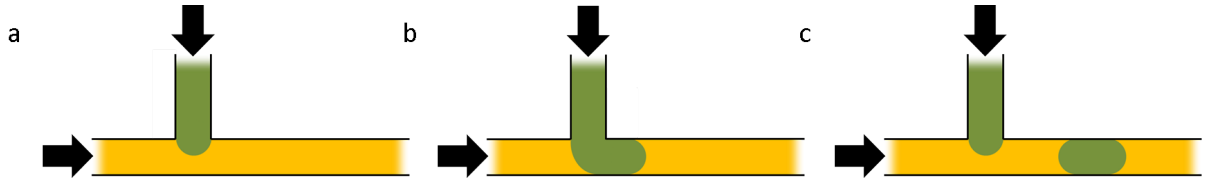


Figure 4: *"Slug" production using continuous cross-flowing stream technique. (a) Aqueous solution (green) is flowed into perpendicular oil (yellow) flow; (b) aqueous column enters oil flow; (c) oil flow pinches aqueous flow creating a slug (droplet in contact with all four walls of the channel).*

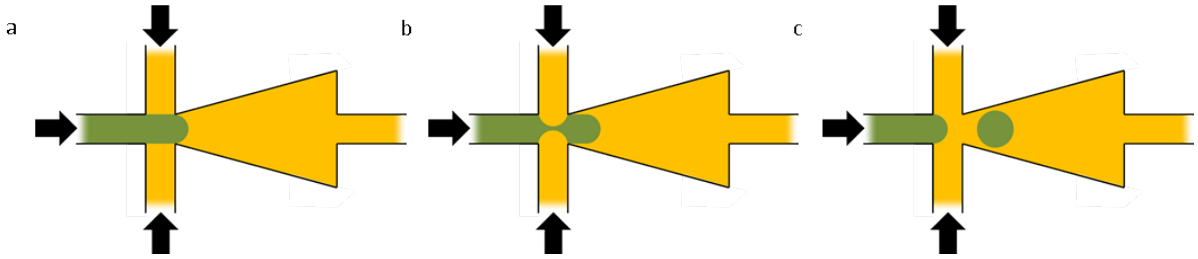


Figure 5: *Droplet production using "flow focussing" technique. (a) Aqueous solution (green) is flowed into the meeting point between two oil phase (yellow) streams; (b) the oil flow pinches the aqueous column at the fluidic "crossroads"; (c) the droplet is separated from the aqueous stream. The droplet formation is enhanced by the diffuser structure, which slows the fluidic flow due to the enlarged cross-section of the fluidics, causing the front of the droplet to slow during formation, giving a smaller droplet size than would have otherwise been achieved.*

Due to the oscillation characteristics of super-excitory BZ media in droplets discussed previously, it appears necessary for the droplets used in this project to be reasonably large. The majority of droplet generation devices found in the literature produce droplets in the tens to hundreds of microns in diameter, often continuously at rates of several thousand droplets per minute. The predominant fabrication method is soft lithography, where poly(dimethylsiloxane) (PDMS) is moulded upon microfabricated photoresist patterns. This now ubiquitous microfabrication and prototyping procedure, first developed by the Whitesides group at Harvard University[10], is outlined in *Figure 6*.

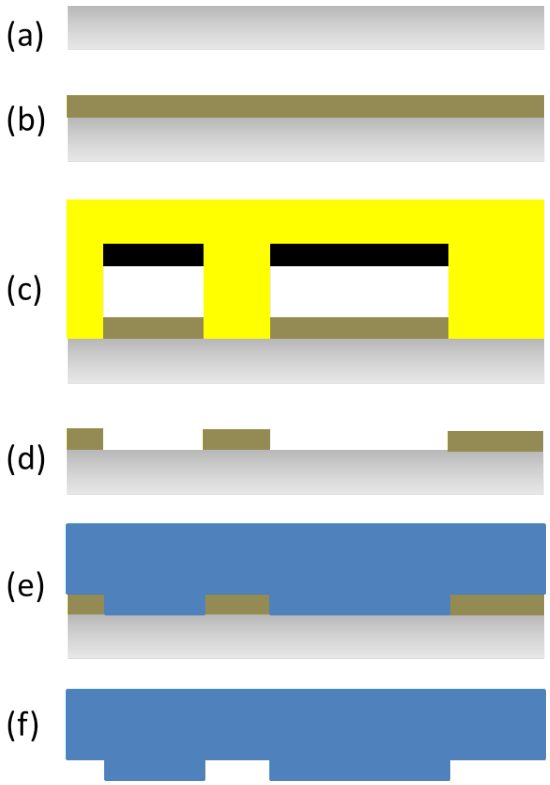


Figure 6: *PDMS-based soft lithography technique. (a) A silicon or glass wafer substrate is prepared; (b) a photoresist material e.g. Su-8 is spun onto the wafer at a defined thickness; (c) the photoresist is patterned by UV light through a photomask; (d) the resist is developed using solvents – in the case of this negative resist, the unexposed photoresist is removed; (e) PDMS monomer is mixed with a curing agent, poured over the surface of the photoresist master and cured in an oven; (f) the PDMS is peeled from the master mould. It can then be bonded to a glass substrate, or another slab of PDMS, via oxygen plasma bonding, creating microchannels defined by the voids in the PDMS and the separate glass/PDMS substrate.*

For our system however, droplets would need to be in the range of 0.5-3.0 mm in diameter, possibly larger. Maximum mould depths achievable in photoresist materials are limited to around 250-500 μm, and then only by using multiple photoresist coats. Additionally, although low-aspect ratio structures can be produced using soft lithography in PDMS, with X/Y axis features unlimited in size, these structure in PDMS tend to be unreliable, as the PDMS is flexible enough to collapse down onto the glass substrate. It was originally decided that direct manufacture of the fluidics would give better high-aspect ratio channels, more suitable for this project.

Towards the aim of large droplet-on-demand, a test rig was produced similar in operation to that found in Churski *et al.*[11] The system comprises of a number of Harvard Pump 11 Plus syringe pumps, with Lee Co 2-way valves used to precisely control the fluid flow into fabricated microfluidic chips. Using this test setup, droplets are produced by closing the aqueous valve, running the syringe pump for a given volume to pressurise the pump-side of the fluidics, and then opening the valve to release the pressure into the microfluidic device. The aqueous valve can then be closed and the process repeated for the next droplet.

This system gives a number of advantages over continuous droplet production methods. Droplets can be produced accurately in small quantities on demand. Furthermore, the size of the droplet is forced by the pre-pressuring of the pump-side fluidics, allowing large droplet production if so desired. This forcing of the droplets also decreases the importance of the fluidic geometries and continuous phase flow rates, allowing a wider range of droplet sizes to be produced from the same generator.

Initial prototype designs of basic microfluidic droplet generators based on the cross-flowing streams (t-junction) concept were produced directly using an Objet Connex350™ 3D Multi-Material printer.[12] The operating principles of this additive layer manufacture (ALM) system are shown in *Figure 7*. The major advantage of ALM over lithographic microfabrication techniques is the ability to produce much higher aspect ratio structures. As it was found that only BZ droplets over 500 μm diameter would oscillate, larger fluidic structures would be required for droplet generation than those found in the literature.

Figure 7: *Operating principles of the Objet Connex350™ 3D Multi-Material printer. (a) The printer nozzles (piezoelectric) deposit a thin but uneven layer of the acrylic material where specified by the 3D CAD design; (b) the fresh uneven material is flattened into a 32 μm thick layer by a precision roller; (c) the fresh material is cured and bonded to the previously deposited material by a high-intensity ultraviolet (UV) light source.*

The structures required are designed using SolidWorks 2009 3D CAD (computer aided design) software package[13], exported into the industry standard .stl file format, before being sliced into the required layers by the Objet proprietary software.

Structures fabricated using this form of ALM are limited by the requirement of support material for overhanging structures, as demonstrated in *Figure 8*. As each layer is sequentially deposited via the printer nozzles, any internal voids need to be filled with support material, otherwise the “ceilings” required will not resolve correctly. Although the Objet software automatically fills such voids with the support material, allowing correct structure resolution, the support material must then be removed. This is normally achieved using a high-power water jet, which is suitable for the removal of external material but will normally only compact material in the small-scale internal voids required for fluidic chips. Additionally, the opacity of even the “translucent” FullCure® material is too high, although it is hoped recently developed materials will overcome this barrier.[14]

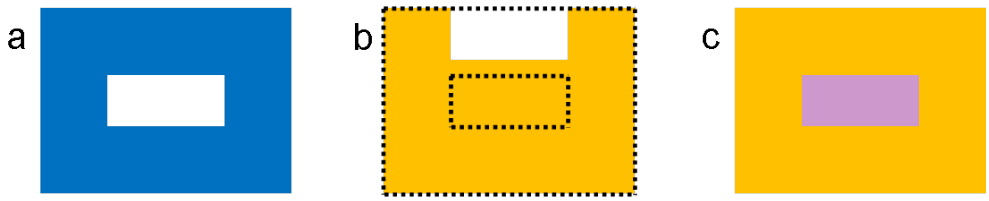


Figure 8: *Production of internal voids in 3D-printed fluidic structures. (a) The required structure, shown from the*

side, to be printed from bottom-up; (b) the resultant structure printed without support material; (c) the correct structure printed with support material filling this internal void, allowing the fabrication of the channel top, but requiring the subsequent removal of the support material.

An initial fluidic chip was produced to try and avoid these problems, as shown in *Figure 9*. The majority of the internal fluidics were left open, to be later covered by a pair of glass slides, thus solving the problems of support material removal and fluidic visibility. However, it was found that the bonding of the glass slides to the 3D-printed structure was non-trivial, due to the roughness of the 3DP material and the tendency for any adhesive used to block the fluidic channels. It was also found that the 3D-printed structure was warped during its removal from the Objet system, further complicating the bonding process. After a number of prototypes, direct manufacture of the devices was dropped in favour of the rapid production of moulds for PDMS.

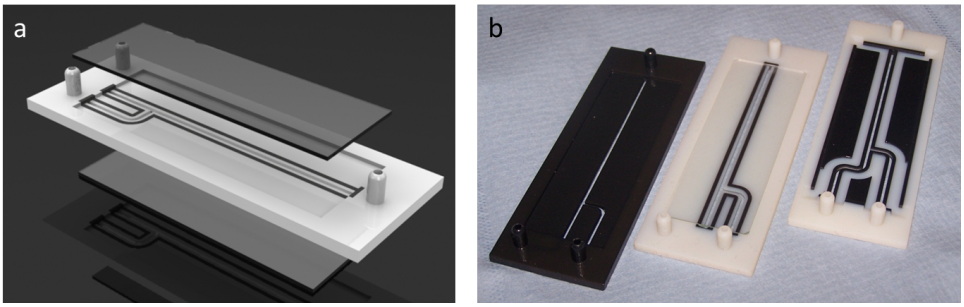


Figure 9: *(a) Original 3DP-produced directly manufactured droplet generator prototype (3D CAD render); (b) various prototypes produced, including designs with integrated rubber gaskets. This direct fabrication approach was abandoned in favour of an indirect manufacture technique of producing moulds for PDMS.*

Rapid production of microfluidic moulds for PDMS using 3D printing:

After the problems found with direct manufacture of microfluidic devices using the 3D printer, it was decided instead to use the system to rapidly produce thin moulds for use with PDMS. This hybrid technique has a number of advantages over more traditional lithography techniques for the purposes of our project, including rapid turnaround time and the production of high aspect-ratio structures. These advantages are found at the expense primarily of final structure resolution (the Object Connex350™ has a final structure tolerance of around 100 μm), although the fluidics for the production of larger droplets do not require the high tolerances of lithographic techniques.

A number of problems were encountered during early production runs with this technique. Early moulds were found to have a surface-related inhibitory effect on the curing of PDMS. It has not been established what the cause of this effect was, but initial casts taken from 3DP-produced moulds had a thin layer of uncured material coating their surface, effecting final feature quality. It was found that an additional baking step (3 hours at 80°C) of the mould before casting with PDMS removed the inhibitory effect.

Another issue was identified where the removal of the 3DP component from the Objet system would induce a curve into the mould component. This would lead to the moulded PDMS not

being flat, mechanically breaking the adhesion to the glass substrate after the oxygen plasma adhesion step. As the 2-hour baking step to remove the surface inhibition raises the mould above the 3DP-material's glass transition temperature (49ºC) it was found leaving the mould on a flat surface during this baking step would flatten the component. The mould was then cooled and attached using PDMS as an adhesive to a glass slide to maintain the flat aspect.

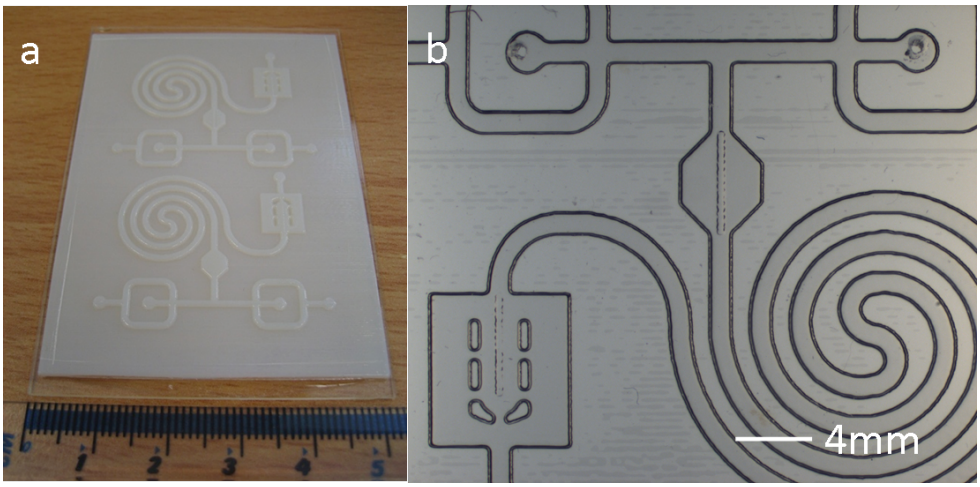


Figure 10: *(a) 3D-printed mould fabricated using the Objet Connex350™ 3D printer, adhered to a glass slide using PDMS; (b) micrograph of resultant channels and microfluidic structures, produced in PDMS and O2-plasma bonded to a glass slide.*

Current fabrication procedure:

1. 3D CAD file produced using SolidWorks 2009;
2. Design printed using Objet 3DP system;
3. Mould removed from system and support material removed using pressurised water;
4. Baking of mould for 2 hours at 80°C;
5. Attachment of mould to glass substrate using PDMS as adhesive;
6. Baked for 1 hour at 80°C to cure PDMS;
7. Mould is silanised using PTFE-functionalised silane;
8. PDMS mixed and degassed, poured over mould in foil boat and degassed again;
9. PDMS cured for 1 hour at 80°C;
10. Cured PDMS removed from mould, cut to size and inlet/outlets punched;
11. Surfaces of PDMS and glass slide O2 plasma treated and press together to seal;
12. Baked at 80°C for 30 minutes to assist adhesion;
13. Device cooled and then silanised to make glass hydrophobic.

Cross-flowing streams for droplet production:

Early designs fabricated using a cross-flowing stream geometry for slug production were found to exhibit problems with the absorption of decane by PDMS, leading to a change in the channel geometry and therefore slug size over time. This was partially solved by reducing the channel height from 1.0 to 0.5 mm, and may in future be eliminated entirely by using a different continuous phase and/or a less permeable device material. Furthermore, it was found to be

difficult in practice to maintain a constant slug size, a problem exacerbated by the production of gas (assumed to be carbon dioxide/monoxide) by the BZ medium in bulk.

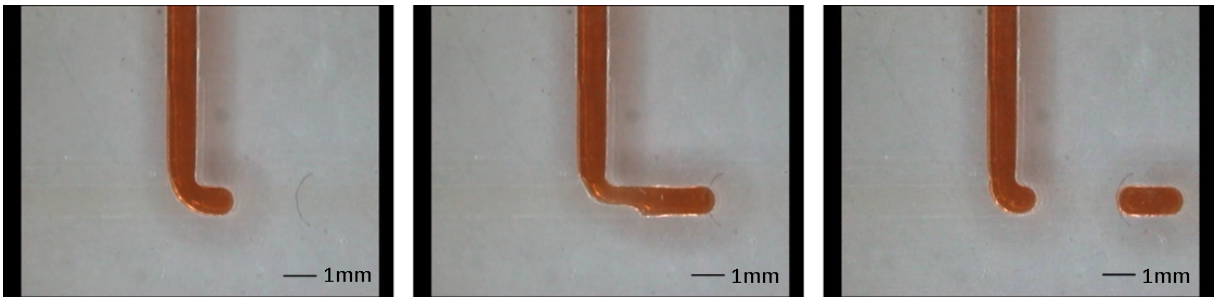


Figure 11: *Continuous production of BZ droplets using a cross-flowing stream device fabricated in PDMS, using a continuous phase of decane with asolectin.*

Flow-focussing for droplet production:

An alternative droplet production method found in the literature is a “flow-focussing” device. These devices inject the aqueous phase into the intersection of a pair of continuous-phase inlets, a process that can be aided by the use of a diverging diffuser element immediately after the phases meet.

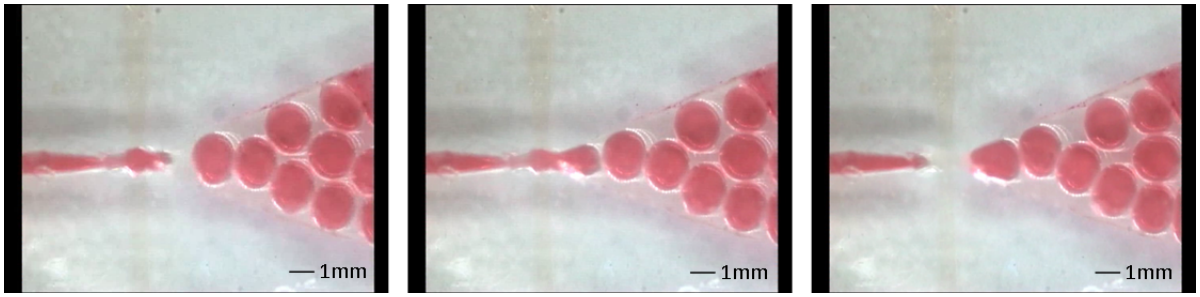


Figure 12: *Continuous flow-focussing of aqueous phase (red food dye) in decane with asolectin, producing droplets of c. 2 mm diameter. The fluidic channels are 1.0 mm wide, 0.5 mm deep, and the diffuser diverges at 18° from the centreline.*

Although this technique is normally used in a continuous-flow mode to produce large numbers of droplets, it has been found that used in conjunction with the system outlined above droplet-on-demand is also possible. It was found that the geometries of the flow-focussing device produced more consistent droplets than those produced using the cross-flowing streams technique. The reason for this is not clear, but it may be that the cross-flowing streams device is more affected by the expansion of PDMS over time.

Subsequently, the flow-focussing device was pared with the valve-based droplet-on-demand system described above. It was found that this setup allowed the production of droplets of a range of sizes, typically between 1 and 3 mm in diameter, without varying the oil flow rate. *Figure 13* shows a pair of droplets produced on the same device, their size varied by changing the pre-pressuring of the aqueous valve.

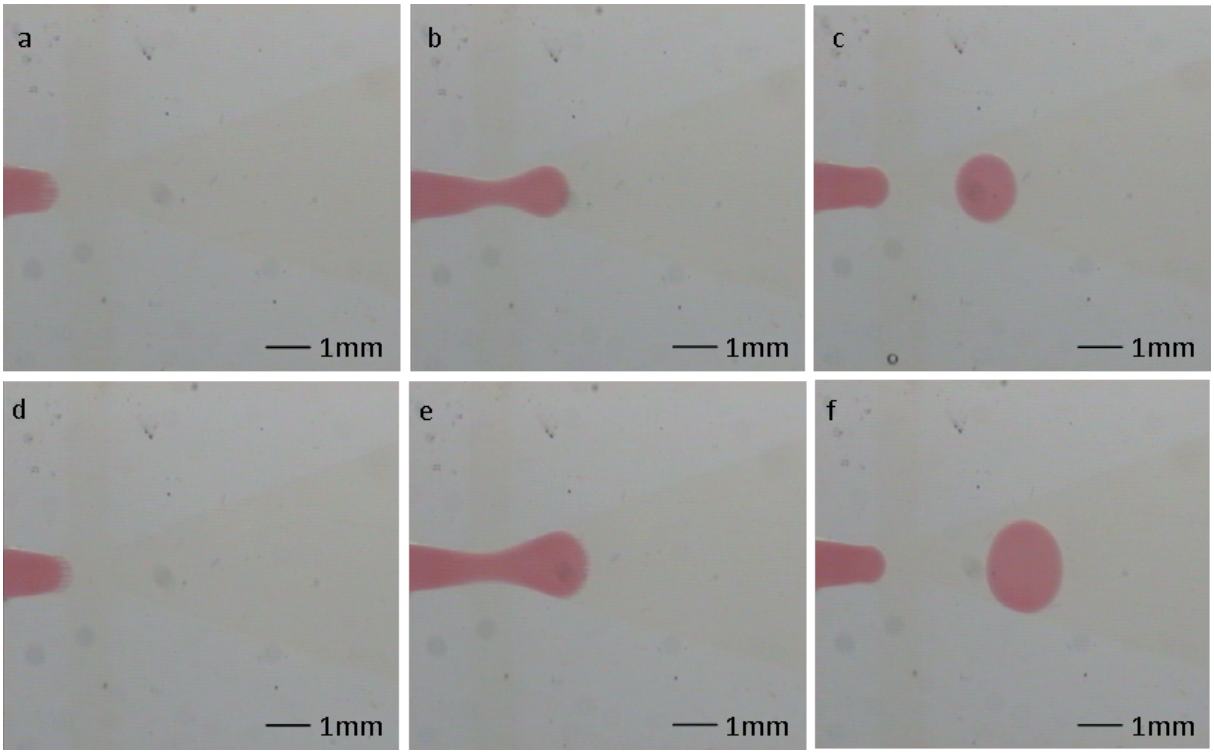


Figure 13: *Production of forced size droplets in a flow-focussing device, with droplets of red food dye being formed in a continuous phase of 60 mg per ml asolectin in decane flowing at 100 µl/min (per oil inlet). A 2-way valve was pre-pressured with a given volume* via *a syringe pump. The valve was then opened and closed to release the pressure and create the required drop on demand. (a-c) On-demand production of 1 µl droplet; (d-f) on-demand production of 2.5 µl droplet. The continuous phase flowrate was identical for each droplet. Note that both droplets attach quickly to the side wall of the diffuser structure rather than travelling down the centre of the cavity.*

Passive control of droplets using the "droplet-on-rails" technique:

In a recent paper, Abbyad *et al*[15] presented a passive technique for the control of droplets in microfluidic channels. This “droplet-on-rails” technique involves the creation of trenches on the floor of the microfluidic device, which the lipid-encased droplets preferentially adhere to due to a combination of surface interaction and lipid surface energy effects.

A test device was prototyped using the same design as shown in *Figure 12*, but incorporating a 300 x 300 µm trench into the ceiling of the diffuser. It was found, as shown in *Figure 14*, that the produced droplets would adhere to the trench, before travelling down the centre of the diffuser along the trench. This is in contrast to the behaviour seen in *Figures 12 & 13*, where the droplets would stick to the side-walls of the diffuser.

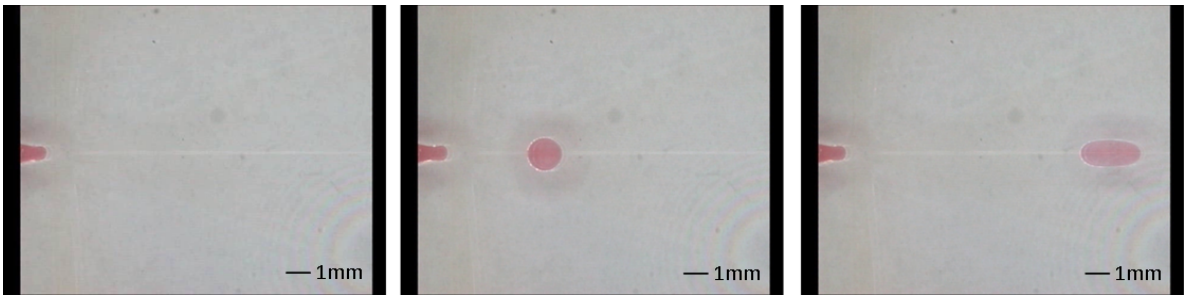


Figure 14: *"Droplets-on-rails" proof-of-concept device, using the droplet-on-demand system described in the main text. The aqueous phase is food dye; the continuous decane with asolectin. Channel dimensions are as described in Figure 2, with the addition of a 300 µm trench to the ceiling of the diffuser channel. The droplets shown are around 1.5 mm in diameter.*

Further devices have been produced to probe more advanced control of the droplets using this technique, including the splitting of droplet streams. However, of more immediate interest is the trapping of droplets into linear arrays for future use in characterising droplet-to-droplet BZ excitation wave propagations. Towards this goal, a structure was produced which allows the capture of 2 mm diameter droplets in a line defined by both a trench and a series of pillars formed in PDMS. Such structures have been previously outlined in the literature, but only to capture small numbers i.e. 1-4 droplets at a time 16-18. The holes between the pillars allow the continuous phase to push the droplets into the area defined by the pillars, without the structure becoming blocked by the incoming droplets, as shown in *Figure 14*.

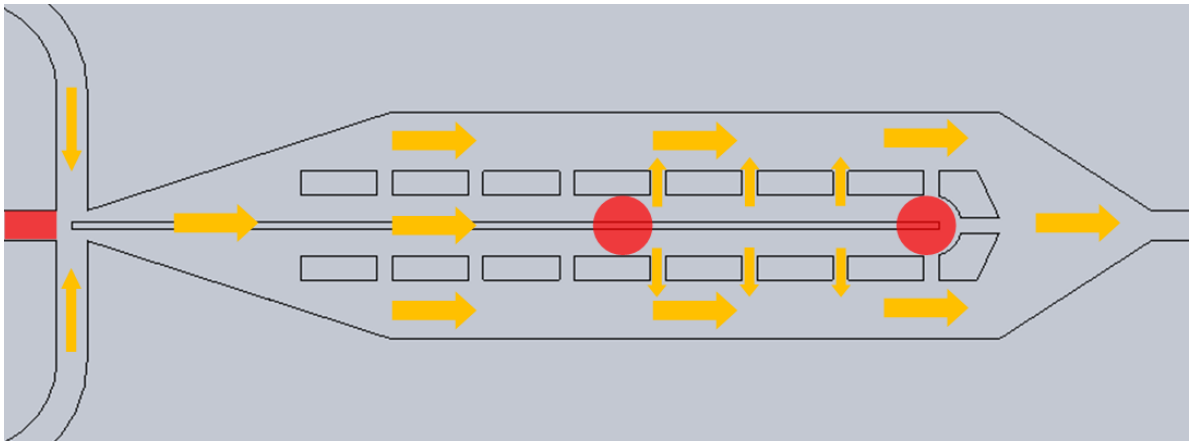


Figure 15: *Concept of droplet capture microfluidic device. Flow focussing device (far left) design is as described previously. The aqueous phase is shown in red; the direction of continuous phase flow is shown in yellow. An initial droplet is captured at the end of the capture area (right-hand droplet), and is followed by a second droplet (centre of image). The second droplet is carried by the oil phase flow, which is not blocked by the first droplet due to holes in between pillars of PDMS that span from the ceiling to the floor of the microfluidics.*

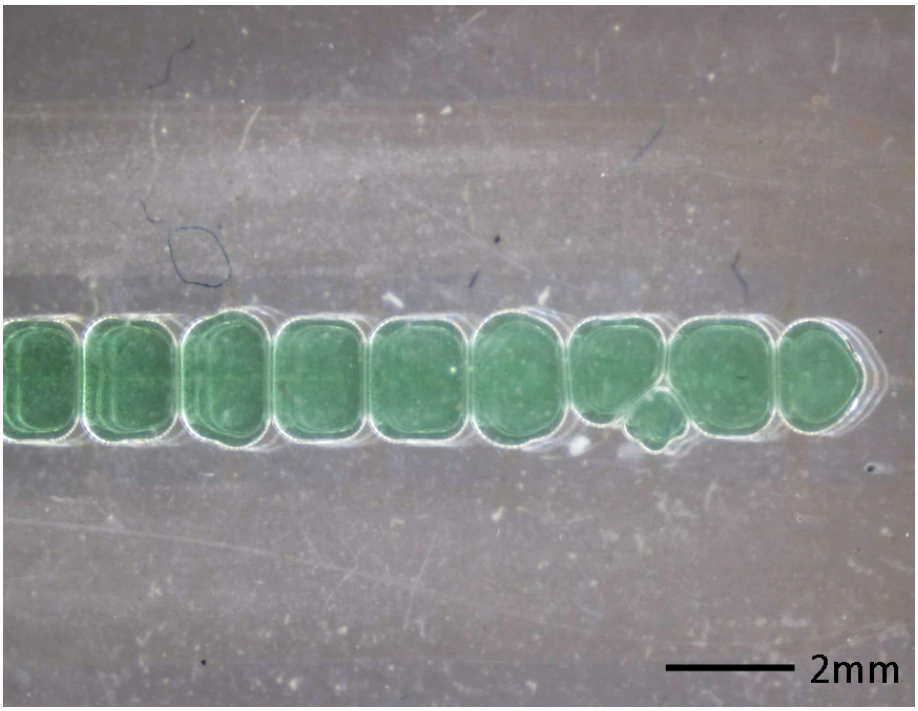


Figure 16: *Proof-of-concept of the droplet capture microfluidic device. Aqueous phase is green food dye; continuous phase is decane with asolectin. The pillars containing the droplets are unfortunately not easily visible. The smaller droplet visible towards the right of the droplet line is due to a miss-firing of the 2-way valve. The droplets are 2 mm in diameter.*

A proof-of-concept is shown in *Figure 16*. The next challenge is to selectively activate individual droplets containing BZ media whilst they are held in the linear array, in order to investigate droplet-to-droplet interactions. This is most likely to be achieved using silver electrodes, although there are a number of barriers to be overcome towards the goal. Another potential experiment involves replicating the work of Stanley *et al*[19], where the formation of droplet interface bilayers was probed by the diffusion of a membrane-permeable fluorescent dye between adjacent droplets.

Merging droplets:

Work has been carried out towards the merging of separately-produced droplets on chip. This work was initiated as a reaction to the problems encountered with the production of CO2 gas by the malonic acid-based BZ reaction when mixed in bulk. However, it is now thought that the cyclohexadione-based media developed recently may solve this problem. However, research into droplet merging has continued, towards the production of droplets with varying concentrations of reactants on chip.

Although there are a number of papers in the literature detailing droplet merging techniques 20, the majority either use no, or very little, surfactant in their continuous phases, promoting droplet fusion. An initial device design was produced, as shown in *Figure 17*, where droplets were brought together at a fluidic t-junction. This design was found to work for the size droplets required for this project, but not reliably enough. It is though that both the swelling of the

PDMS, and possibly differences in the ionic strength and/or viscosity of the dyes used in these experiments may cause differences in the droplet formation time, leading to the droplets sometimes not meeting at the t-junction at precisely the right time. It is hoped in future work that this system will be refined to allow the production of droplets on demand with variable chemical content.

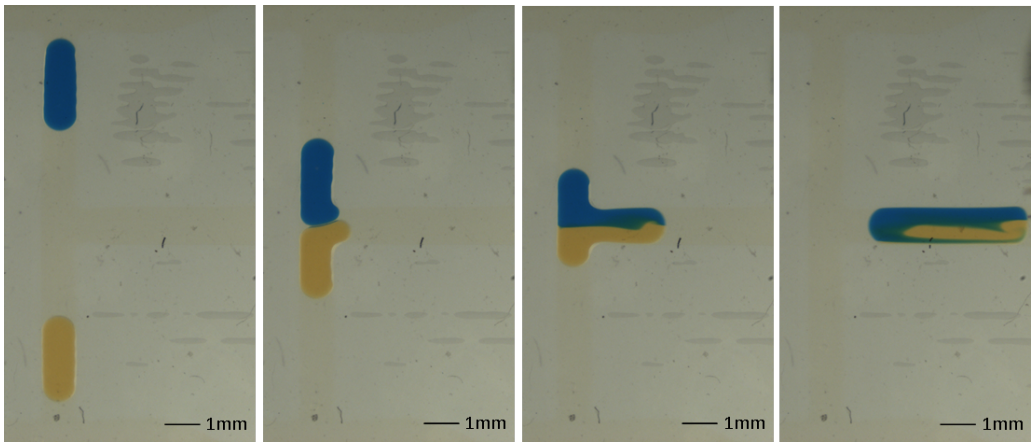


Figure 17: *Initial droplet merging prototype microfluidic chip. Aqueous phases were yellow and blue food dye; continuous phase 60 mg per ml asolectin in decane at 100 µl/min flowrate (per droplet generator i.e 200 µl/min at t-junction). Droplets are seen to come into contact, merge and then start to mix, as demonstrated by the green colouring produced.*

Beyond PDMS:

It is becoming increasingly apparently that PDMS is not an ideal material for the production of BZ droplets in oil. Not only does the absorption of the oil phase into the PDMS lead to material swelling, distorting the microfluidic structures, but PDMS is not acid-tolerant, as shown in *Figure 18*. Although BZ had been used previously directly in PDMS microchannels e.g. as shown in *Figure 11*, mixing the medium from separate components naturally requires higher component concentrations, leading to more obvious damage over a shorter time period. This is compounded by the observation that CHD-substrate BZ droplets produced on a PDMS chip do not oscillate, and instead park in the oxidated state. It is not known why this is so, but it may well be due to the leaching of BZ constituents from the droplets into the PDMS, or the contamination of the droplets by compounds present in the PDMS.

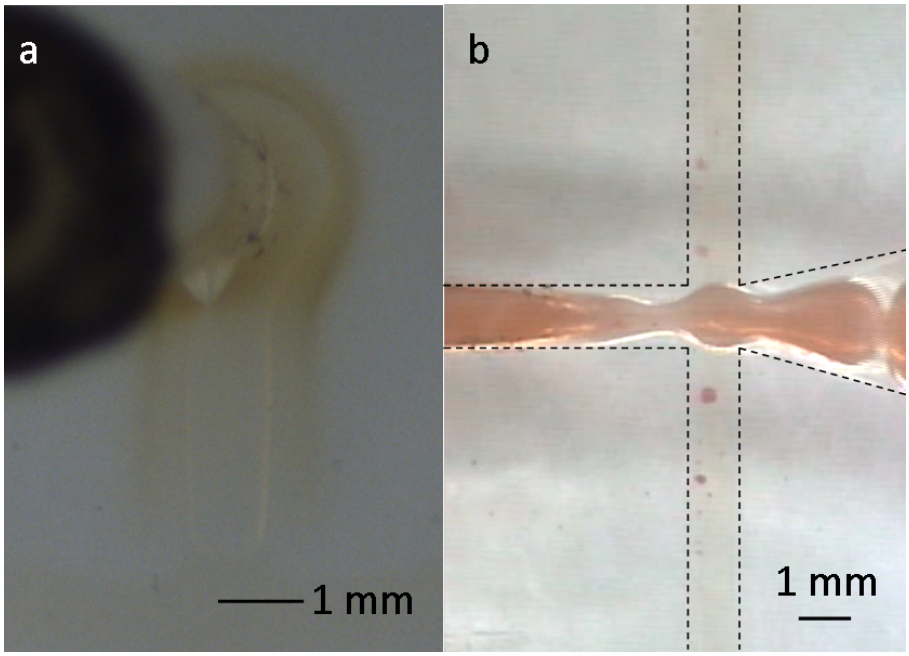


Figure 18: *(a) PDMS inlet to a microfluidic chip, containing fluid column of H2SO4 0.623 mM and malonic acid 0.225 mM, towards production of BZ media on-chip. Damage (yellowing) of the PDMS in a zone around 500 µm deep can be seen around the acid column. It was also noted that the acid solution was yellow on withdrawal from the device, indicating the leaching of damaged PDMS compounds into the solution; (b) Swelling of PDMS due to absorption of decane, most visible in the "nozzling" of the aqueous solution to the right of the fluidic junction of this flow-focussing device. The device fluidics were 1000 µm square in cross-section - the height was reduced to 500 µm in future devices to reduce this problem. Dotted lines show the original fluidic dimensions after fabrication.*

A promising avenue of future research is the production of microfluidic chips using fluorinated polymers. A recent paper[21] detailed the use of 3M Dyneon™ THV Fluorothermoplastic. This terpolymer of tetrafluoro ethylene, hexafluoro propylene and vinylidene should offer exceptional non-polar solvent and acid resistance, and work has begun into the processing steps required for working with this polymer.

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