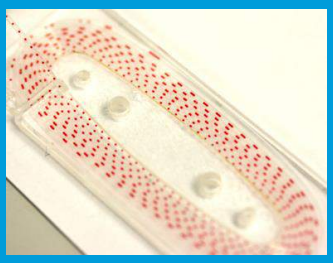
Mitos Dropix® Droplet Generation System

Demonstration of Droplet-on-Demand Sequencing (Mode 1)



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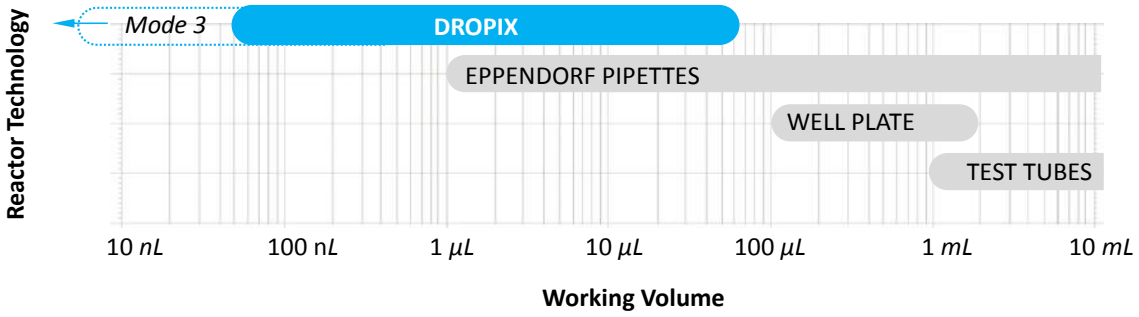
Mitos Dropix® Technology

There is increasing demand for screening massive numbers of biological reactions, increased speed of screening and reduced reagent consumption. In large scale research, the bulk of the cost is attributed to reagents and screening libraries. Traditional liquid handling systems address the throughput and speed requirements, but have limitations on working volume, especially below 1µl. This is particularly important for applications which require screening of high value or scarce reagents. Current microplate technologies operate on 1 – 100 µL volumes, and there is considerable interest in scaling assays down to nL or pL volumes to reduce the costs of reagents.

Droplet-based assays are particularly well suited for high throughput screening owing to their ability to operate in miniscule volumes. As a relatively new technology, there are presently no commercial liquid handling systems utilizing droplet technology. Mitos Dropix® technology now introduces liquid sampling and processing over a very wide 10 nL – 50 µL volume range utilizing droplet technology.



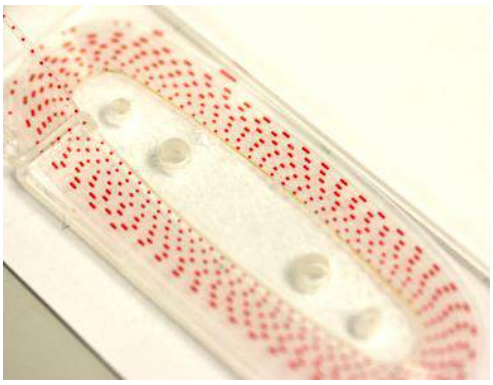
10^6 reduction in working volume between Mitos Dropix® and various commercially available reactor technologies. A description of Modes follows in the next section.

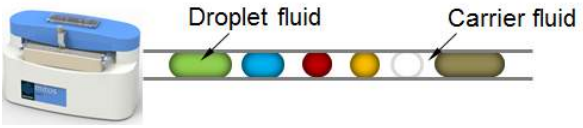
In drug discovery and the fields of biology and chemistry, it becomes necessary to screen hundreds-of-thousands of compounds for high-throughput screening applications. Droplet systems have proved to be effective for many types of screens, including cell assays, ratiometric screening for protein crystallization, directed evolution of enzymes, etc. Mitos Dropix® therefore enables droplet based high throughput screening in synthetic chemistry, fundamental biology and pharmacology amongst other disciplines. This application note demonstrates the droplet sequence production for library generation using simple fluids to help readers understand the concept, and to be able to assess its applicability to specific lab protocols.

Mitos Dropix® was developed by Dolomite under exclusive sub-licence with Drop-Tech Ltd. having won Dolomite's 2012 Productizing Science® competition. Drop-Tech was formed from an academic collaboration between Cambridge University and Imperial College London and is the exclusive licensee of their patented droplet generation technology used in Mitos Dropix® **(Patent Pending: PCT/GB2013/051668).**

Abstract

Droplet-on-demand sequencing and droplet library generation is demonstrated with the Mitos Dropix® in this application note. Its ability to create nanoliter droplet sequence with programmable volume and composition (out of a maximum of 24 fluids) is demonstrated. Using simple fluids, the capabilities are shown to be extensible to a much wider variety of fluids, relevant to users of many disciplines. Continuous operation is demonstrated whereby the fluids are refilled during operation without affecting performance. Additional design features of the enable process integration with 192 well-plate format liquid handling systems.





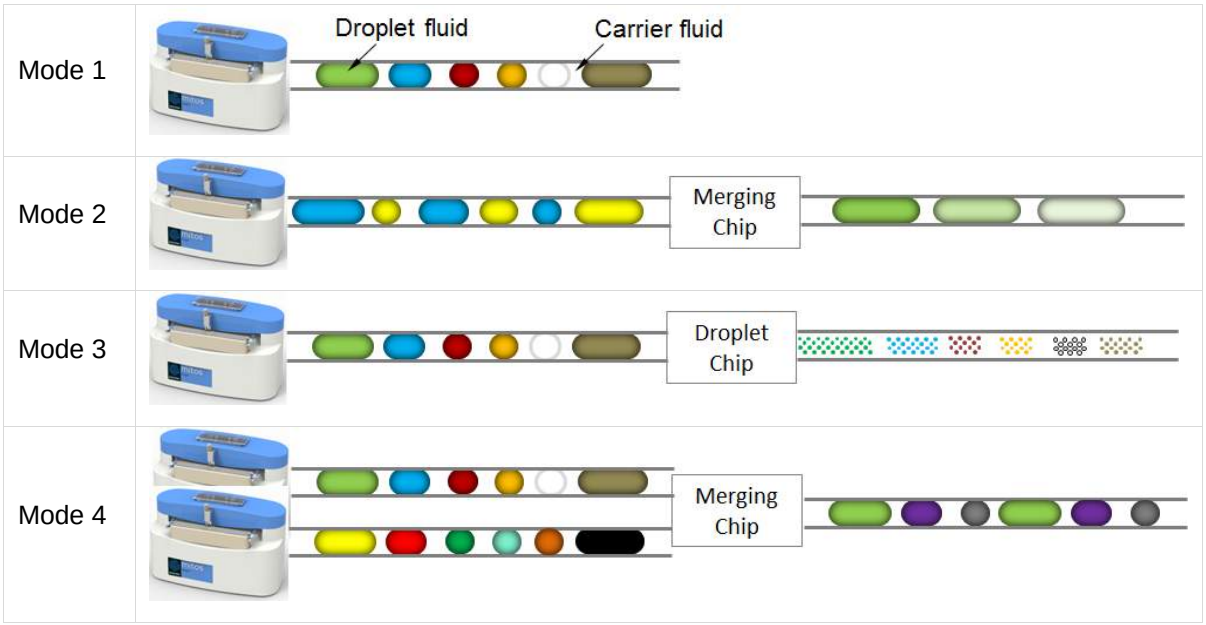
In a test case, a droplet sequence of 6 different aqueous droplets spaced by FC-40 carrier fluid was produced in 50 consistent iterations. A library of approximately 2000 aqueous droplets was created with 6 variable compositions in a 2 hour period. Bright field imaging is used to visualize and characterize droplet sizes. Droplets with volumes as low as 30nL and as high as 1000 nL were produced. The largest possible droplet size is the maximum volume of sample loaded in the sample strip which is 50 µL. An accuracy of ±10% was achieved over the entire dynamic range.

Exploiting fluid surface tension and buoyancy are key aspects incorporated in the Mitos Dropix® technology. Ensuring accuracy with fluids over a wider range of properties is enabled with the provision of a user specific calibration feature. Detailed method for custom calibration, system part numbers, and useful FAQ's are presented as Appendices.

It is shown that Mitos Dropix® delivers droplet-on-demand for a range of volume and composition. Using the software interface, production is shown to be automated and programmable. Its underlying principle of droplet-on-demand is universal to research labs across the globe.

Modes of Operation

Four basic modes of operation are suggested. These may be further customized to suitspecific requirements. The general setup involves the use of one or more Mitos Dropix®, a pumping system and a microfluidic device such as a storage coil, a droplet chip or a merging chip. These four modes are illustrated below.



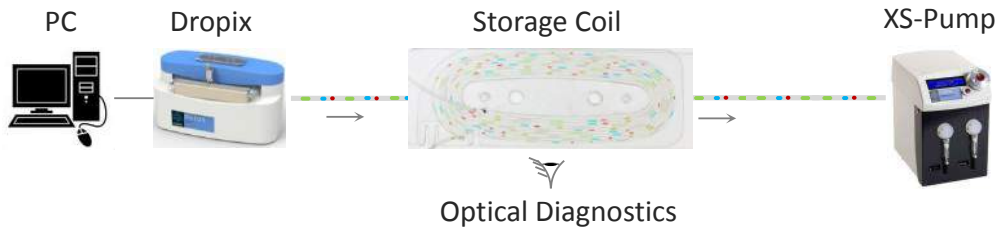
4 basic modes.

- Mode 1: Droplet sequence production or droplet library generation. Applications requiring a large reference droplet library are able to utilize this mode. A single Mitos Dropix® has the capacity to store 24 different fluids, and therefore the library consists of 24 different compositions.
- Mode 2: Mixing or concentration gradients from 2 or more wells using a merging chip. Utilizing a droplet merging chip, ratiometric mixing from up to 24 wells, results in effectively infinite droplet combinatorial reactions.
- Mode 3: Either in-line with mode 2, or independently, Mode 3 extends Mitos Dropix® from nanoliter technology to picoliter technology. This is most effectively achieved with the use of a droplet production microfluidic device.
- Mode 4: Synchronized multiple Mitos Dropix®. Applications requiring more than 24 starting solutions will benefit from using multiple Mitos Dropix®.

Setup

In this application note, droplet-on-demand sequencing and library generation is demonstrated with the Mitos Dropix® setup in Mode 1. Six aqueous samples were loaded into the Mitos Dropix® and a sequence of nanoliter volume droplets was generated with preset volume and composition. To distinguish between the different water samples, food dye was added to each sample before it was loaded into the Dropix®Sample Strip. The volume of each water sample was 50µl. The Dropix®Fluid Reservoir - PMMA (Part No. 3200414) was filled with FC40 which is an inert and biocompatible fluorocarbon oil. The Mitos Dropix® was connected to a Mitos Duo-XS Pump which aspirated the droplet and carrier stream into the Droplet Storage Coil. A sequence of droplets was generated, separated by the carrier oil. The XS pump, due to its dual syringe function, has the added benefit of constantly refilling the carrier fluid bath during operation, thereby maintaining a steady fluid level.

Appendices carry information on system configuration, helpful hints, FAQ's and a general note about droplet-on-demand applications. A droplet volume calibration method is also described.



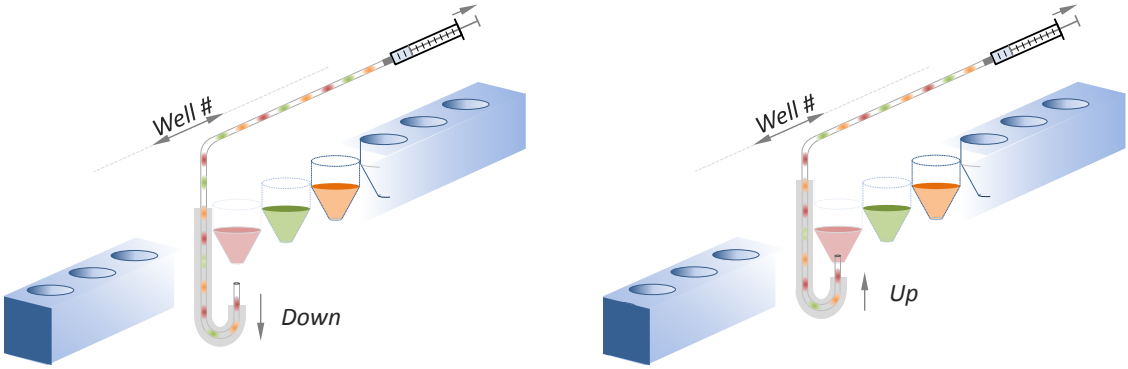
Schematic of setup required for making droplets in sequence. Arrows indicate direction of flow – the syringe pump works in withdrawal mode.

The system setup is shown in the figure above. Fluidic connections between the Mitos Dropix® (Part No. 3200350), Droplet Storage Coil (Part No. 3200349), the Dropix®Sample Hook (Part No. 3200353, 3200355) and the XS-Pump (Part No. 3200057) are made using FEP tubing of OD 0.8 mm and ID 0.25 mm (Part No. 3200302). Flangeless Ferrule 0.8mm, ETFE (Part No. 3200306) and End Fittings and Ferrules for 0.8mm Tubing (Part No. 3200307) are useful for making connections with the pump. Visualization was achieved using a DSLR camera with a macro-lens and brightfield imaging.

The syringe pump continuously aspirates a stream of droplets from the Mitos Dropix®, gradually filling the storage coil. The flow rate set on the syringe pump was 5 µl/min. It is important to check that there is no outgassing or cavitation in the syringe pump while it is running. The Mitos Duo XS-Pump provides a pulse-free flow which is essential for consistent and accurate droplet formation.

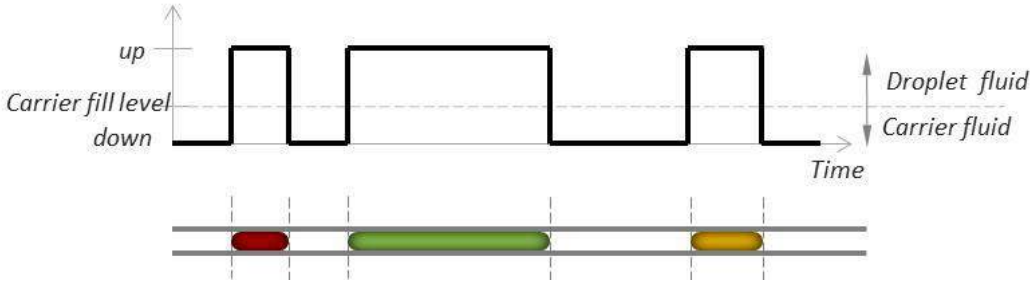
The diagram below explains the operation of the Mitos Dropix®. The Dropix®Sample Hook assembly has two vertical positions:

1) Down position – in this position the carrier fluid, oil, is aspirated.
2) Up position – in this position the aqueous sample is aspirated, creating the droplet.



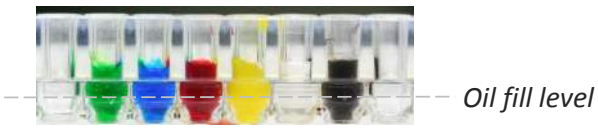
Schematic showing Dropix®Sample Hook assembly motion relative to the Sample Strip. Left: In 'down' position aspirating carrier fluid. Right: In 'up' position asiprating droplet fluid.

The Dropix® Sample Hook assembly also moves from 'left' or 'right' to allow selection of the sample by travelling to the target well. The timing of the 'up' and 'down' movement controls the droplet volume and carrier spacing respectively, as shown below:



'Up' and 'down' Motion of the sampling nozzle controls droplet size and carrier spacing.

Starting with a fresh sample strip, 6 (out of the 24 wells) were filled with 50 µl of water with food colouring as show below (see appendix for helpful hints on well filling).



Sample holder and sample strip with 6 (out of 24) wells in use. Aqueous reagents loaded in 6 wells – Well#12 (green), Well #13 (blue), Well #14 (red), Well #15 (yellow), Well #16 (clear), and Well #17(brown). Buoyancy and surface tension help retain the sample in the well.

The Dropix® Sample Strip (Part No. 3200351) was then removed from the Dropix® Sample Strip Holder (Part No. 3200356), and carefully placed onto the Dropix® Fluid Reservoir - PMMA on the Mitos Dropix®, as shown below. Filling the Dropix® Sample Strip outside the Dropix® Fluid Reservoir - PMMA works well in most cases. If the oil or samples have a high surfactant concentration then this can result in low surface tension which can occasionally cause the sample to fall out of the wells. To resolve this issue,

place the Dropix® Sample Strip into the Dropix® Fluid Reservoir - PMMA before loading the samples.

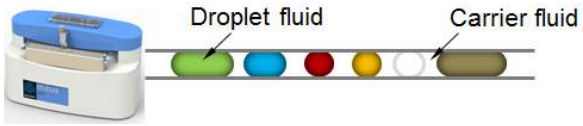
Left: Dropix® Sample Strip Holder with multiple Dropix® Sample Strips in a 192 well format. These are filled using a micropipette. Right: Sample Strip transferred from the Dropix® Sample Strip Holder onto the Dropix.

The Dropix® Sample Strip is disposable and designed around the format of a 192 well plate. The Dropix® Sample Strip Holder enables interfacing with other fluid handling automation.

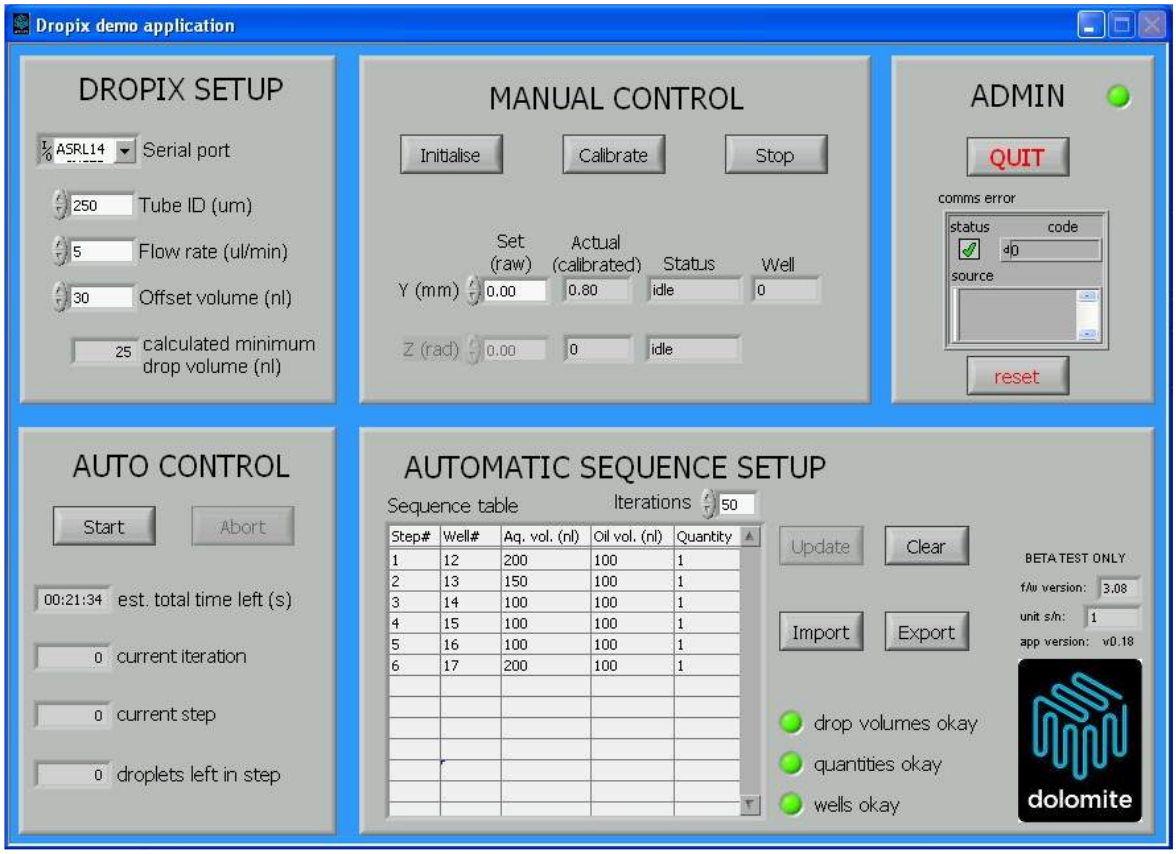
During the experiment, the oil level in the reservoir drops as a result of the oil carrier fluid being aspirated by the pump. It is therefore necessary to top up the oil on a regular basis.

Fill level indicator in the carrier fluid reservoir. The carrier fluid can be refilled during operation.

The image below shows the droplet sampling sequence which was setup in the Dropix® software. A six droplet sequence is input with droplet sizes in nanoliters and the volume of the carrier fluid in between droplets. The iterations, or number of repeat sequences, was set to 50 (max of 255). Detailed information on operating the software is available in the user manual.



The droplet sequence and the droplet volumes were set in the Dropix® software, which also allows users to modify and recall droplet sequence programs.



Dropix® software setup to process 6 different reagents from 6 wells. A single Mitos Dropix® has capacity to carry up to 24 different reagents.

Results

As the Dropix® Sample Hook assembly rises in a well, the tube orifice transfers from the carrier fluid into the droplet fluid while the syringe continually aspirates. The target droplet volume translates into timing for the rise and fall of the Dropix® Sample Hook assembly based on an inviscid fluid assumption.

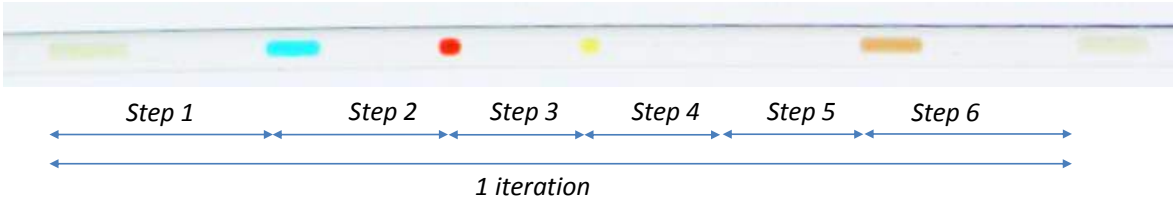
Real life fluids being viscous, and surface tension dominated at low volumes, there is a small offset that creeps in. This appears due to a delay between the time at which the hook crosses the interface, and time at which the droplet fluid is drawn into the tubing. The tubing being hydrophobic carries around it a film of organic fluid, which must drain prior to aspirating aqueous fluid. The droplet fluid being non-wetting with the FEP tubing, never contacts the tubing, thereby completely supressing cross-well contamination.

The difference between the target volume and the aspirated volume is expected due to deviation from inviscid to viscous flow, and is managed conveniently by means of Mitos Dropix® calibration. Calibration is advised to achieve improved accuracy and is described for a simple situation in detail in Appendix A. In this case, the calibration offset was found to be 50 nL. The offset between target droplet volume (set in the software) and actual

droplet volume aspirated (measured) is expected to be constant. The size of the offset varies between different fluid systems as it is affected by fluid viscosity and fluid surface tension.

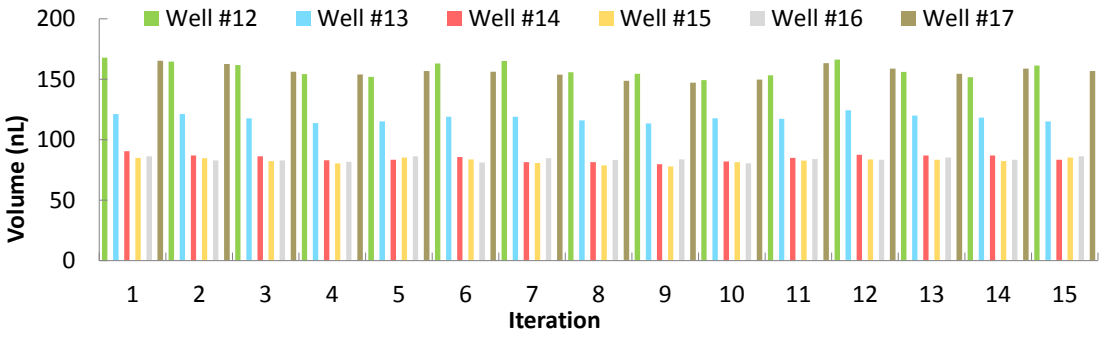
As the droplets flow through the tubing, and into the flexible FEP tubing, they can then be imaged. Images of the droplets were recorded using a DSLR camera with macro-lens and brightfield imaging. The droplets appear as elongated slugs in the semi-transparent tubing. The length of the slugs were measured to estimate droplet volumes.

The below figure shows 7 droplets – green (200 nL), blue (150 nL), red (100 nL), yellow (100 nL), clear (100 nL) and brown (200 nL). The software was setup to produce 50 iterations of this sequence. The fifth droplet contains no dye and is not visible in the image below. The seventh droplet is the repeat of the first, and indicates the start of the next iteration.



Bright field image of sequenced droplets in a section of tubing. The above program will produce 50 such iterations. Droplets travel left to right at 5 µL/min in an FEP tubing of ID 250 µm and OD 800 µm.

The above sequence was recorded as a video which was broken into frames allowing measurement of droplet size. The volume of droplets was then calculated and plotted over time as shown below.

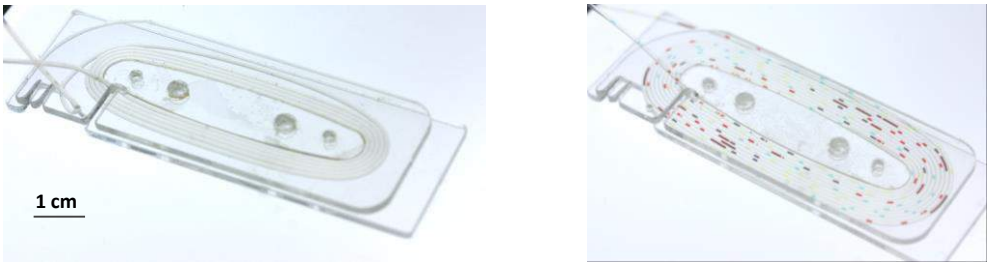


15 repeat steps of sequencing 6 reagents over approximately a 5 minute period. Target volumes are: green (200 nL), blue (150 nL), red (100 nL), yellow (100 nL), clear (100 nL) and brown (200 nL).

To demonstrate consistency over iterations, the above data is presented in terms of the variation relative to the target droplet volume. This is indicative of the dispersity of the droplet size. The subset of droplet sizes in the above test are 100, 150 and 200 nL, performance results of which are presented in the below table.

Target Droplet Volume (with offset included)	Number of droplets in test	Measured droplet volume (average of 15 droplets)	Variation of droplet volume around target for 15 droplets	Variation of droplet volume around target for 15 droplets
100 nL (150 nL)	15	103 nL	Max = 110 Min = 97 100nL -3/+10	-3%/+10%
150 nL (250 nL)	15	138 nL	Max = 145 Min = 134 150nL -16/-5	-10%/-3%
200 nL (300 nL)	15	178 nL	Max = 188 Min = 170 200nL -30/22	-15%/-7%

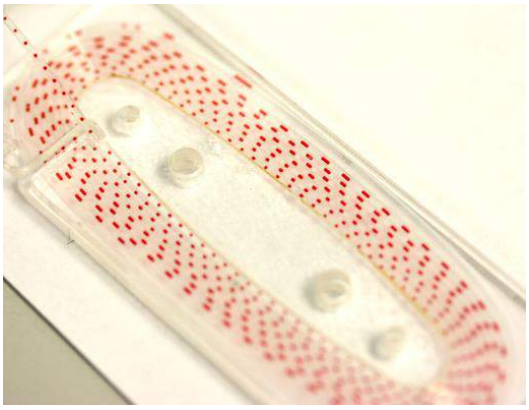
The droplets were collected in the Droplet Storage Coil. This coil holds an FEP tube with an O.D. of 0.80 mm, I.D. of 0.25 mm and a length of 1.0 meters. As the FEP tubing is semi-transparent, the Droplet Storage Coil can be used as an observation cell for optical diagnostics such as brightfield or fluorescence microscopy. The Droplet Storage Coil can also be placed in an incubator. Safe temperature limits are available in the user manual.



Left: Empty storage coil made of FEP tubing of 0.8 mm OD. Right: Nanoliter volume sequenced droplets ready for push/pull operation.

Conclusion

The Mitos Dropix® system was assembled, tested and droplet sequencing (also known as droplet-on-demand) was demonstrated successfully. Six 50µl aqueous samples were loaded into the Dropix® Sample Strip and converted into a sequence of nanoliter droplets in a fluorocarbon oil carrier stream. The range of droplet volumes generated was 50nl – 1000nl. The variation in droplet volume for a set of 10 samples was typically within ±10% around the target value. This result includes a fixed volume offset of 50nL which is independent of flow rate and droplet volume. It does however depend on the viscosity and surface tension of fluids.



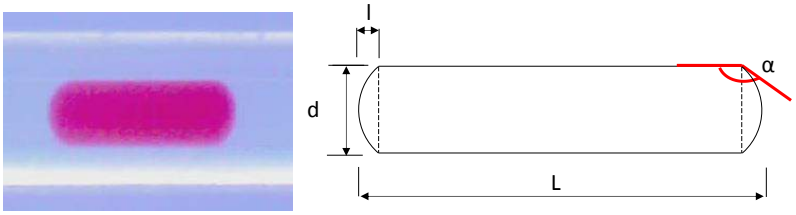
A Droplet Storage Coil with ~ 450 reactions in a 3 inch^2 area. The fluid flow rate sets the bounds of throughput.

The Dropix® Sample Strip can hold 24 aqueous samples, each with a maximum volume of 50 µl. Each 50µl aqueous sample can be split into between 50 - 1000 droplets based on a droplet volume range of 50nl – 1000nl. It is possible to refill the sample strip during operation without significant disturbance to droplet production.

Appendix A: Mitos Dropix® calibration

The calibration method is described below in steps, and then demonstrated in greater details for a single aqueous fluid sample. The sample is loaded into the Dropix® Sample Strip, droplets of which are made in an immiscible FC-40 carrier fluid. When using multiple samples, it is recommended that the average of the calibrations be used in the setup.

1. Program the Mitos Dropix® to produce a range of droplet volumes and carrier volumes
2. Measure the droplets lengths and calculate the droplet volume using the equation shown below:



Left: Image of droplet. Right: Approximation of droplet shape incorporating contact angle.

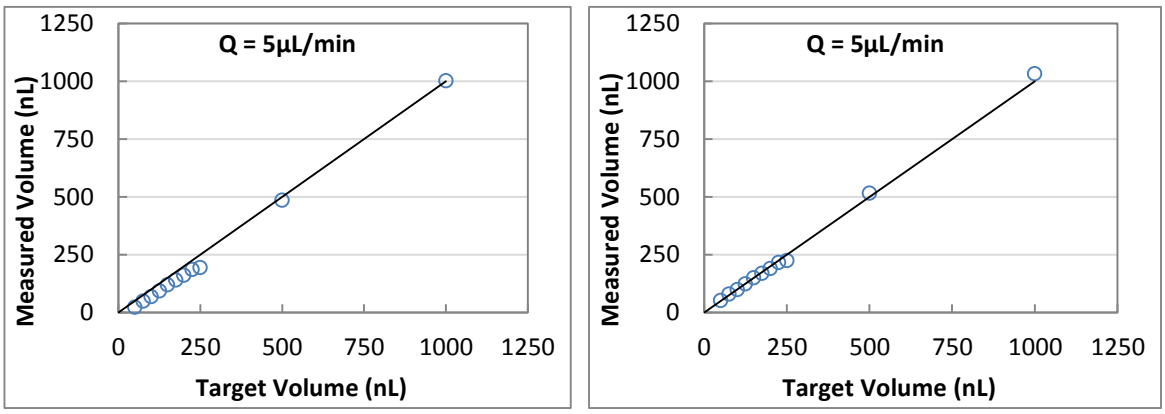
In most cases, the contact angle is not 180°, and the end caps deviate from a hemi-spherical shape. The droplet volume is then given by:

Volume of end cap	$V_{cap} = \frac{\pi}{3}\left(-\frac{d}{2\,cos\alpha}\right)^3 (2 - 3sin\alpha + sin^3\alpha)$
Length of end cap	$l = \frac{d}{2}\,tan\alpha - \frac{d}{2\,cos\alpha}$
Volume of cylinder	$V_{cyl} = \pi\left(\frac{d}{2}\right)^2 (L - 2l)$
Total volume of droplet	$V_{tot} = V_{cap} + V_{cyl}$

Note: FEP tubing typically has an internal diameter of 0.25mm ± 0.0125mm (+/-5% tolerance). Droplet volume estimation will be accurate to ±10%. The tubing may be calibrated by injecting a 10µl water droplet into a dry or oil filled tube and measuring the length of the slug. If the length is 203.8mm then the tubing I.D. is accurate. If it is different then a tubing I.D. calibration factor should be applied.

3. Compare the measured droplet volumes with the with the target volumes. Typically the aqueous droplet volume will be below the target volume by a fixed offset and the carrier fluid volume will be above by the same amount.
4. Calculate the offset based on averaged results.
5. Adjust the offset volume in the setup section of the Dropix® software.

The graph below (on the left) shows the uncalibrated results (without the offset) and the graph on the right shows calibrated results (with the offset). In the tests the droplet volume was increased from 50nl to 1000nl while the carrier volume between droplets was held at 100 nl. The actual droplet volumes were consistently 30nl less than target volumes with the water-FC40 oil fluid combination.



Q = 5 µL/min. Left: Uncalibrated. Right: Calibration with offset = 30 nL.

In the above graph, data points lying on the line indicate a condition of equal measured volume compared with target volume. If the data point deviates from the line, this is an indication of requirement of calibration.

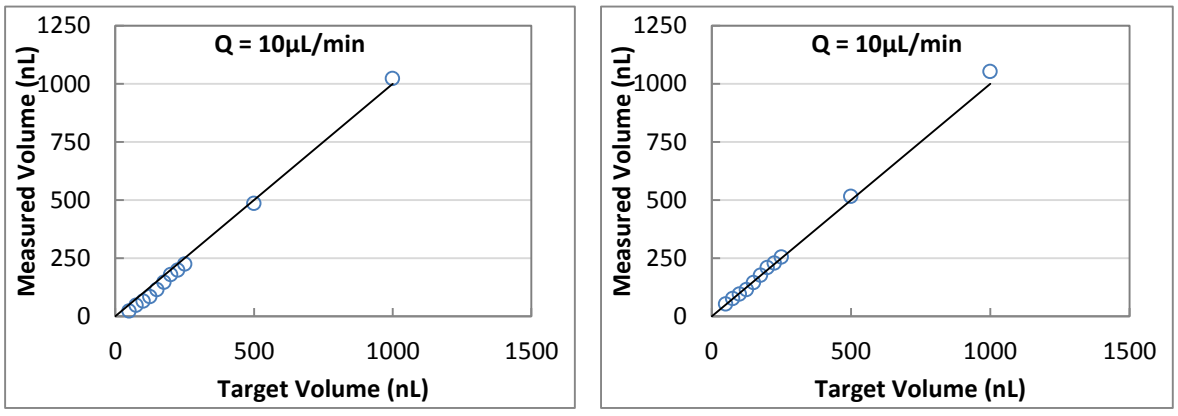
The results shown above were collected in an experimental run with duration of 2-3 hours during which time ~2000 droplets were collected. The graph shows that the offset remained constant with the droplet volume being consistently 30nl less than the target

value and the carrier volume being 30nl higher than the target. During this period the sample wells and reservoir were refilled several times. This did not significantly affect the sample volume. The number of droplets required depends on the fluid selection, and is preferably higher in case of large dispersity in size.

The table below shows the results along with the variation of the droplet size for 10 samples of different volumes:

Target Droplet Volume (including offset)	Number of droplets in test	Measured droplet volume (average of 10 droplets)	Range of droplet volume around target for 10 droplets	Range of droplet volume around target for 10 droplets
50 nL (80 nL)	10	51 nL	Max = 56 Min = 43 50nL +6.3/-6.3nL	-12.6%/+12.6%
100 nL (130 nL)	10	87 nL	Max = 97 Min = 83 100 nL -2.5/-16.5nL	-16.5%/-2.5%
250 nL (280 nL)	10	226 nL	Max =237 Min =216 250 nL -13/-33.3nL	-13.2%/-5.2%
500 nL (530 nL)	10	517 nL	Max =527 Min =509 500 nL +27.9/+9.3nL	1.9%/+5.6%
1000 nL (1030 nL)	10	1031 nL	Max = 1045 Min =1020 1000 nL +45.2/+20.2nL	2.0%/4.5%

The test was repeated at a higher flow rate and found to be unchanged.



Q = 10 µL/min. Left: Uncalibrated. Right: Calibration with offset = 30 nL.

Target Droplet Volume (with offset included)	Number of droplets in test	Measured droplet volume (average of 10 droplets)	Variation of droplet volume around target for 10 droplets	Variation of droplet volume around target for 10 droplets
50 nL (80 nL)	10	50 nL	Max = 53 Min = 47 50 nL -2.5/+3.5nL	-5.0%/+7.0%
100 nL (130 nL)	10	97 nL	Max = 108 Min = 87 100 nL -12.99/+8.5nL	-12. 9%/+8.5%
250 nL (280 nL)	10	229 nL	Max = 239 Min = 221 250 nL -28.1/-10.9nL	-11.2%/-4.3%
500 nL (530 nL)	10	501 nL	Max = 525 Min = 480 500nL -19.7/+25.1nL	-3.9%/+5.1%
1000 nL (1030 nL)	10	1049 nL	Max = 1060 Min = 1037 1000nL +37.9/+60.6nL	+3.8%/+6.1%

Appendix B: Mitos Dropix® System Component List

Part No.	Part Description	#
3200350	Mitos Dropix®	1
3200197	USB to RS232 Adaptor Cable	1
3200349	Droplet Storage Coil – 0.25mm	1
3200414	Dropix® Fluid Reservoir - PMMA	1
3200351	Dropix® Sample Strip (Pack of 8)	1
3200356	Dropix® Sample Strip Holder	1
3200353	Dropix® Sample Hook – 0.8mm	1
3200355	Dropix® Sample Hook Fitting – 0.8mm	1
3200302	FEP Tubing, 0.8mm x 0.25mm, 10 metres	1
3200306	Flangeless Ferrule 0.8mm, ETFE (pack of 10)	1
3200307	End Fittings and Ferrules for 0.8mm Tubing (pack of 10)	1
3200057	Mitos Duo XS-Pump	1
3000252	Syringe for Mitos Duo XS-Pump, 1ml	2

3000245	Valve for Mitos Duo XS-Pump (3 Port)	2
3200050	High Speed Camera and Microscope System	1

Appendix C: FAQs & Helpful Tips

Q: What is the correct position of the FEP tube?

A: Slight adjustment is possible to the position of the tubing and is useful if the fluids are unusually high viscosity. The tubing may be extended beyond the position of the calibration up to a maximum of 1 mm. Care should be taken, as excessive extension may cause the tubing to scrape the bottom of the wells in its transverse motion.

Q: What is the best method to fill the wells?

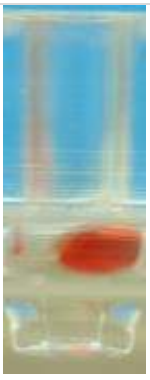
A: The method of fluid filling dictates the position of the interface. Using a micropipette, gently fill the wells with the required volume. Ensure that no air bubbles are trapped in the well or along the walls of the well. If required, empty out well and refill.

Q: Is it necessary to prefill the sample holder before installing?

A: The Dropix® Sample Strip can be installed into the oil-filled Dropix® Fluid Reservoir – PMMA with the wells either full or empty. Although it is recommended to fill the wells beforehand, this is not critical.

If sample is filled into the wells after installation, the interface position is dictated more by buoyancy than by pinning of the interface to the narrowing mouth of the well bottom. Although this is less preferred, this option is attractive for low surface tension fluid systems.

Q: What is the best indicator on whether or not the well is filled properly?

				
Correct – interface is flat, no air bubbles	Incorrect – air bubble stuck near mouth	Incorrect – aqueous fluid too high in well	Incorrect – aqueous fluid too low in well	Incorrect – aqueous fluid risen up in the well

A: The aqueous fluid should rest at the mouth of the wells as in the image to the left. To the right, a well is seen with an air bubble lodged, a pendant droplet hanging, or with a receding interface – these are undesirable and require corrective action. Adjustment with a micropipette evens out the hanging drops. There should be no air bubbles visible at the

bottom of the well, in the tubing, or anywhere in the fluidic system. Good priming is essential for proper control. These may be affected by the surfactant content in the fluid.

Q: What is the start-up duration?

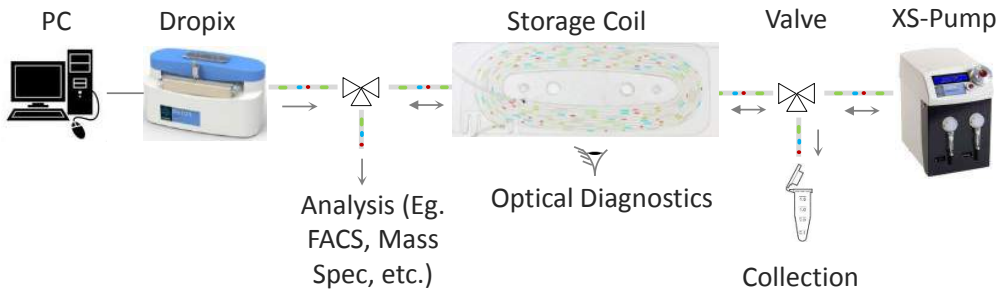
A: Start-up is defined as the time required for the programmed droplets to fill the tubing. After this stage, sample can be analysed.

Q: Can an alternate method of pumping be used?

A: Yes, it is possible to use any method of pumping. A positive displacement pump is best suited as this assures a more reliable flow rate compared with a pressure pump.

Q: Is it possible to use valves to steer the droplet sequence?

A: Yes, the use of valves may cause droplet stability issues, but a push-pull operation may be done with the arrangement shown below.



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