Agarose Encapsulation on the Nadia Innovate

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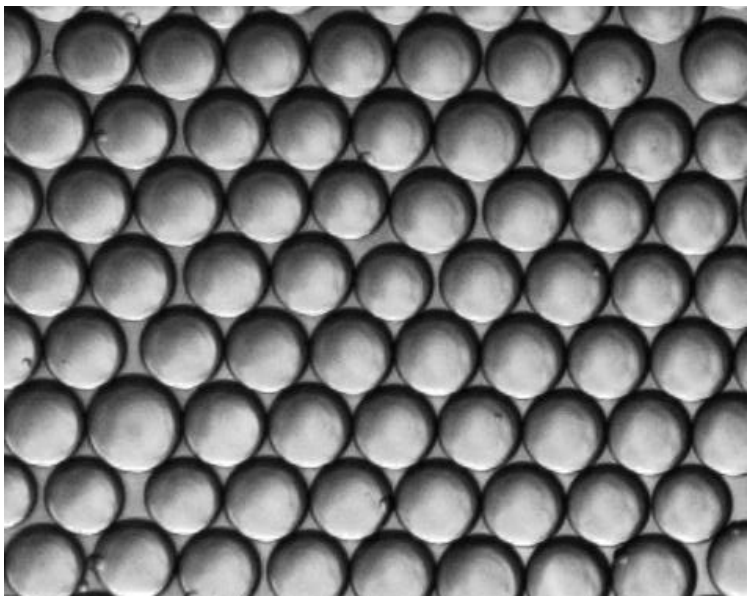

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Summary

This application note describes the formation of agarose microdroplets. It details the steps taken to generate a custom profile for creating and subsequently recovering agarose droplets using the Nadia Innovate, by altering parameters such as pressure, temperature and stirrer speed.

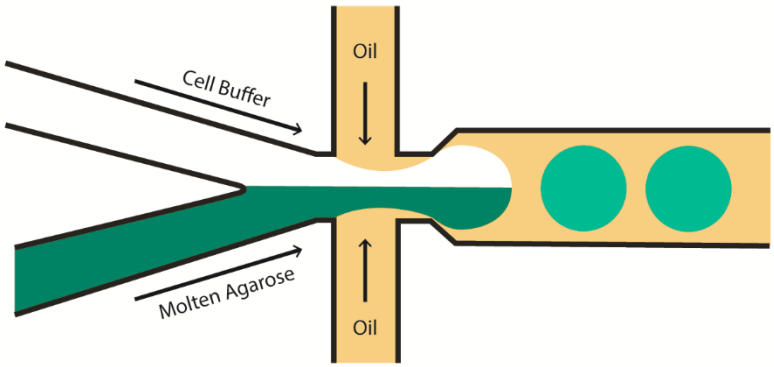


Figure 1 Schematic of agarose droplet generation. Flow focussing allows droplets of agarose to be formed at extremely high throughput. This technique can be used to encase cells or other biological material in a spherical crosslinked scaffold once the agarose has hardened.

Introduction

Hydrogel droplets, such as those made of agarose, are picolitre-volume spherical scaffolds which remain stable in an aqueous solution. They represent a potent solution for many single cell applications [(1)]. Due to the properties of hydrogels which allow the diffusion of nutrients and dissolved gases to circulate and reach the encased cells, agarose encapsulation is a powerful tool that allows cells to be grown in individual microenvironments for extended periods of time.

Conventional plate-based cell culture methods attempt to simulate a cell-growth environment in a two-dimensional plane. In contrast, encapsulating biological material within hydrogel droplets allows cells to be grown within three-dimensional scaffolds, more closely mimicking their native physiological environment. These properties of hydrogel droplets can also facilitate cell seeding onto 3D scaffolds to form hydrogel matrices for *in vitro* tissue synthesis. The generation of highly monodisperse hydrogel droplets in this application is paramount, as the uniform size of droplets enforces their predictable stacking into a geometric tetrahedral 3D structure [(2)]. Hydrogel bead-based cell delivery systems also represent promising vessels for efficient drug delivery. The encapsulation of cells which continuously secrete therapeutics inside hydrogels has clear advantages over microbeads containing only limited concentrations of active drugs. Due to the pico-litre volumes of substrate which single cells can be encapsulated in using droplet microfluidics, co-encapsulating live cells in hydrogel alongside miniscule volumes of active drugs, stressors or growth factors can additionally act as a potent technique for assaying single molecule-cell interactions.

Furthermore, the ability of microfluidic devices, such as the Nadia Instrument, to co-encapsulate two distinct cell types in agarose allows the high throughput analysis of single cell-cell or cell-pathogen interactions over biologically relevant timeframes. This, coupled with the capacity to FACS-sort droplets along with their encapsulated contents, means that agarose encapsulation holds great potential for bacterial isolation and analysis [(3)]. With the in-built temperature controller of the Nadia and Innovate platforms, molten hydrogel can be flowed into droplets as a liquid before being hardened into solid spherical scaffolds. This can be conducted at biologically relevant temperatures anywhere between 1°C and 40°C. The following application note uses the example of agarose droplet generation to demonstrate how the Nadia Innovate can be used to efficiently optimise a protocol for hydrogel encapsulation. From here, the incorporation of single cells into these hydrogel microdroplets represents a potent tool for single cell analysis.

The objectives of the experiments described in this application note were, firstly, to use the Nadia Innovate to define pressure conditions, stirrer speeds and temperatures that allow for stable encapsulation of agarose on a Nadia Innovate chip. Secondly, we aimed to test the recovery of agarose droplets from the resultant emulsion.

Materials and Methods

Droplet system. Dolomite Bio's Nadia Instrument (***Error! Reference source not found.***) is designed to allow high-throughput analysis of single cells and single nuclei using droplet microfluidics. It produces highly monodisperse droplets using three independent pressure pumps. The Nadia Instrument has the capability to heat and cool all reagents between 1°C and 40°C, allowing a variety of novel applications to be pioneered here. Furthermore, runs on the Nadia Instrument can be customised by allowing the user to change sample volume and sample stirrer speed to adapt for different experimental requirements.

With the addition of the Nadia Innovate module, the Nadia Instrument is converted into an open development platform for the optimisation of new applications and protocols. Customisation options are extended to droplet size and generation rate via precise user control over line pressures. Additionally, dynamic temperature and stirring control of samples are centrally accessible via the Dolomite Bio Flow Control software, allowing real-time observation and optimisation of droplet production via the Nadia Innovate high-speed digital microscope.

The Nadia platform uses disposable plastic COC (cyclic olefin copolymer) chips to perform the encapsulation. This prevents reagent cross-contamination between runs and ensures dried agarose within a cartridge does not affect subsequent runs. For the Nadia Innovate, single sample plastic chips are loaded into the Nadia Innovate module and positioned such that the microfluidic junction can be imaged. Once pressure profiles for generating agarose droplets are developed on the Nadia Innovate, the parameters can be transferred to the Nadia Instrument to be run in high throughput. The chips used in this application note feature fluorophilic 80 µm microfluidic junctions capable of encapsulating cells or objects up to 40 µm in diameter.

Figure 2 The Nadia Instrument (right) and the Nadia Innovate platform (left) set up depicting all the components of a Nadia Innovate system.

Agarose. Ultra-low Gelling Temperature agarose (A2576, Sigma-Aldrich) was mixed with water at a 1.5 % concentration and molten in a microwave oven. The agarose was then filtered through a 0.2 µm syringe filter and either loaded immediately or stored at 50 ˚C in an incubation oven for up to 4 hours. Alternatively, the agarose can be stored on a hot plate set to 50 ˚C whilst being constantly stirred by a magnetic stirrer bar. The final concentration of agarose inside the droplets was 0.75%, due to it mixing at a 1:1 ratio with the cell buffer upon encapsulation. The composition of the cell buffer is described below.

Note: Agarose concentrations exceeding 2 % were found to be prone to partial gelling within the microfluidic channel of the Nadia chip, resulting in inconsistent agarose concentrations in the resultant droplets.

Agarose encapsulation. Prior to sample loading and throughout the duration of the run, the TCU was set to 40 °C in order to keep the agarose in a molten state. As per the standardised loading instructions for both the Nadia and Innovate platforms, 3 ml of QX200™ Droplet Generation Oil for EvaGreen (#1864005, BioRad) was loaded into the oil reservoir of the Nadia Innovate chip. 250 µl of 1.5 % Ultra-low Gelling Temperature agarose was pipetted into the agarose reservoir of the Nadia Innovate chip. Note that in the current version of Dolomite Bio Flow Control Centre (FCC) software (3.22.3), this agarose reservoir is referred to as "bead reservoir" due to previous nomenclature from RNA-sequencing applications. To most closely mimic the buffer composition of prospective samples, the cell sample reservoir was filled with 250 µl of a typical cell buffer (1x PBS with 0.01 % BSA). This exemplar cell buffer was prewarmed to 37 °C to avoid sudden cooling and hardening of the agarose solution upon meeting at the microfluidic interface.

Encapsulation parameters were optimized using the Nadia Innovate module and Dolomite Bio FCC software. Pressures, stirrer speeds and temperatures were altered using the FCC software and their effects on droplet generation were assessed in real time using the high-speed digital microscope camera video feed focussed on the microfluidic junction. After the run had ended, the resultant emulsion was transferred from the Nadia Innovate chip output reservoir to a microcentrifuge tube using a P1000 pipette. To harden the agarose beads inside each droplet, the emulsions were then stored at 4 °C for 30 minutes before being processed further. All emulsions and agarose beads were imaged using a Zoe BioImager (BioRad).

Note: Due to the design of both the Nadia Instrument and the Nadia Innovate platform, no integral instrument parts are wetted by reagents throughout the encapsulation process. Therefore, to ready the system for the next run, the disposable Nadia Innovate chip and associated gasket were simply discarded.

Results

Using the Nadia Innovate to optimise encapsulation parameters. Sample loading, pre-run and run temperatures were set at 40 °C to ensure that the agarose solution remained molten for the duration of the run. Due to heat dissipation from the chip, this ensured that the temperature within the cell reservoir was maintained at approximately 37 °C. To further enforce the uniform suspension of the agarose solution, the pre-run stirrer time for the agarose reservoir was adjusted to 60 seconds and the stirrer speeds were set to 200 rpm for the pre-run stage and to 150 rpm during the run. This increased initial stirring time also served to allow heat from the TCU to adequately heat the entire agarose reservoir. Stirrer speeds for the cell buffer reservoir remained at 75 rpm to avoid damage to samples. These parameters are summarised in Figure 3.

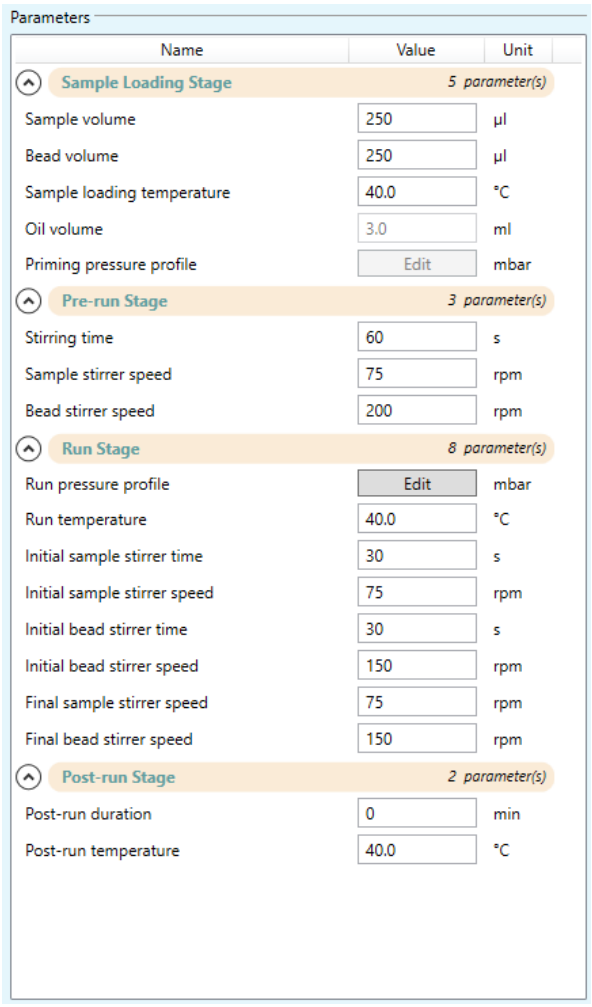


Figure 3 Summary of editable parameters used in this agarose encapsulation application taken from the Dolomite Bio FCC software. Note that in this current version of the software (v3.22.3), parameters affecting the reservoir containing agarose are referred to as “bead”.

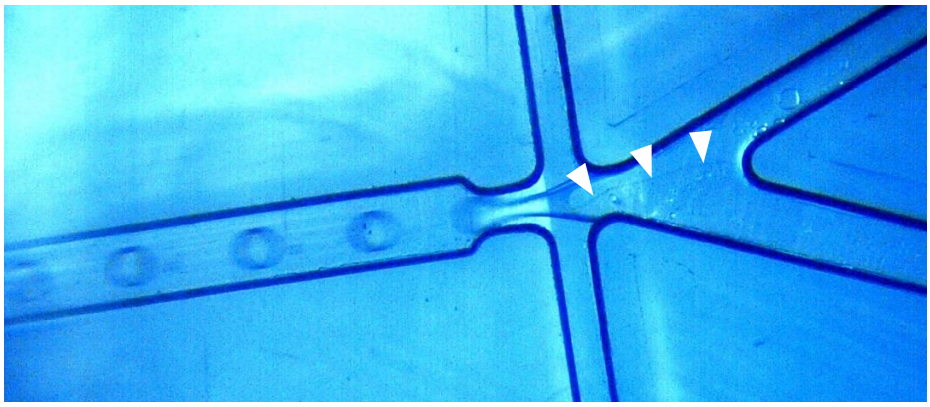
To overcome the increased viscosity of 1.5% agarose compared to other buffers such as PBS, the pressure applied to the agarose line was adjusted to 300 mbar. In addition to these parameters, the pre-run priming steps for the agarose line were set to 300 mbar to ensure that the high-viscosity solution was sufficiently primed up to the junction prior to the start of encapsulation. Following this, the pressures on the oil and cell buffer lines were adjusted to 500 mbar and 150 mbar respectively to ensure stable droplet generation. These values were defined by real-time alteration and live analysis of droplet generation at the junction using the Nadia Innovate high-speed digital microscope. The customised pressure profile generated by these optimisations can be seen in Table 1.

Table 1 Pressure profile for generating 80 µm 0.75 % agarose droplets using the Nadia Instrument. Priming steps, encapsulation steps and post-run steps are highlighted in blue, green and grey respectively. Step pressures which were optimised specifically for this application are highlighted in yellow.

Step #	Step Duration	Oil Pressure (mbar)	Agarose Pressure (mbar)	Sample Pressure (mbar)
1	00:00:01	450	0	0
2	00:00:01	450	40	40
3	00:00:01	450	70	60
4	00:00:05	450	300	100
5	00:00:01	450	300	100
6	00:00:01	450	40	40
7	00:00:01	450	100	400
8	00:00:01	450	100	400
9	00:00:06	450	100	133
10	00:00:01	450	100	133
11	00:00:03	450	140	600
12	00:00:01	450	190	600
13	00:15:00	500	300	150
14	00:00:01	500	300	150
15	00:00:01	450	0	0
16	00:00:01	0	0	0

The finalised Innovate protocol was then run on a new Nadia Innovate chip without further alterations. The generation of droplets at the microfluidic junction is documented in Figure 4(a), and the resultant droplets, after the 30-minute incubation at 4 °C, are documented in Figure 4(b). Droplets demonstrated good monodispersity with an average size of 80 µm and contained semi-transparent agarose beads. This protocol exhausted the cell buffer and agarose in the reservoir within 15 minutes.

(a)



(b)

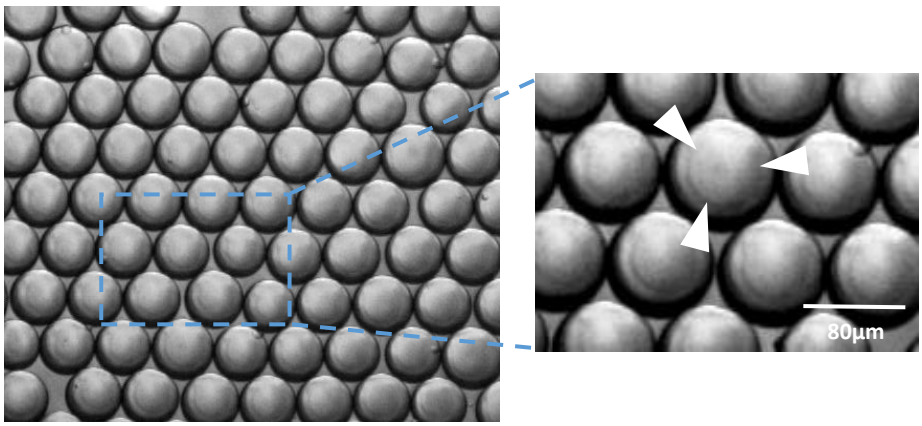


Figure 4 (a) Agarose emulsions were produced and droplet formation imaged at the Nadia Innovate chip junction The interface between cell buffer and 1.5 % agarose solution is visible (demarcated by white arrows). ***(b)*** Agarose emulsions imaged after formation show good monodispersity, with hardened agarose beads being visible inside droplets (an inlaid example of an agarose sphere within an aqueous droplet is highlighted at a higher magnification by white arrows).

After protocol transferral to the Nadia Instrument, the parameters developed using the Nadia Innovate module were used to run 8 sample simultaneously generating approximately 3200 µl of agarose droplet emulsion within 15 minutes. For access to the Nadia Innovate protocol file generated in this application note, please contact applications@dolomite-bio.com

Recovery of the agarose beads. Agarose beads were recovered from the droplets via breaking of the emulsion with perfluorooctanol (PFO) (370533, Sigma-Aldrich) after collection of the available emulsion in a microcentrifuge tube.

Using a standard pipette, as much of the oil suspension as possible was removed from the lower phase within the microcentrifuge tube. This minimised the amount of PFO necessary to break the emulsion. The volume of emulsion was estimated using the graduations on a 1.5 ml microcentrifuge tube. A typical Nadia or Innovate run using the defined pressure profile should yield approximately 400 µl of emulsion. To break the emulsion, two volumes of PFO were added to one volume of emulsion and the tube was inverted five times by hand.

Following emulsion breakage and phase separation, two layers appeared inside the tube, a clear upper layer that contained the agarose beads and a slightly opaque lower layer with the unwanted fluorous phase. The tube was tilted at a 45-degree angle and the aqueous supernatant layer was removed and kept in a clean microcentrifuge tube for further experimentation or analysis. The agarose beads in the aqueous phase were then pipetted, inserted into a haemocytometer chip and observed under a light microscope (Figure 5).

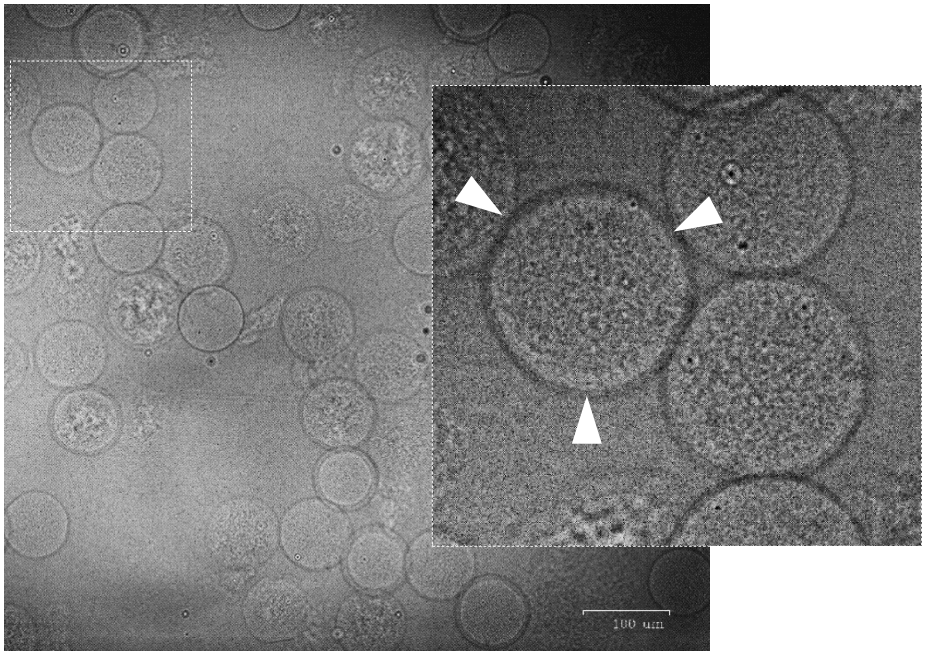


Figure 3 Recovered agarose beads (~80 µm diameter) suspended in an aqueous solution following emulsion breakage. A subset of droplets within the dotted square are magnified in an inset image, with a single agarose droplet being highlighted by white arrows. Due to their near-transparency, high contrast levels are necessary to visualise agarose beads. Additionally, agarose beads do not lie stacked in a flat plane as droplets would, leaving some beads outside of the focal plane of the microscope, giving them a false appearance of having duplicated outlines.

Recovered agarose beads exhibited good monodispersity and an average diameter of 80 µm. Note that due to their porous nature, agarose beads will swell and shrink depending on the aqueous solution in which they are suspended.

NOTE**:** Once the droplet emulsion has been broken and separated from the oil phase, agarose droplets can be FAC-sorted. When producing hydrogel beads for FACS, one might consider using the Nadia Innovate parameter control to increase oil pressure and decrease droplet sizes to ~60um, making them compatible with selective FACS instruments.

Conclusion

Hydrogel droplet encapsulation is a useful tool for the individual culturing or imaging of singulated cells, or even FAC-sorting cells along with their associated microenvironments. This application note showed that by using the Nadia Innovate system, agarose can be readily encapsulated inside droplets and solid agarose beads can be recovered. The beads produced were monodisperse and their size could be adjusted by altering pressures using the Nadia Innovate module.

(1) Luo R-C. and Chen C-H., "Structured Microgels through Microfluidic Assembly and their Biomedical Applications", Soft. 2012 Dec;1;1-23

(2) Dolega M. E., et al., "Controlled 3D culture in Matrigel microbeads to analyze clonal acinar development", Biomaterials. 2015 Jun;52;347-57

(3) Eun Y-J., et al., "Encapsulating Bacteria in Agarose Microparticles using Microfluidics for high-Throughput Cell Analysis and Isolation", ACS Chemical Biology. 2011 Dec;6;260-266

Product Information

Nadia Innovate system

Description	Order Number
Nadia Instrument	3200590
Nadia Innovate	3200595
Nadia Innovate Upgrade for Modular Systems	3200660

Nadia Innovate consumables

Description	Order Number
Innovate Chips – 8 runs (8x1)	3200611
Innovate Chips – 40 Runs (40x1)	3200612
Innovate Cartridge & Droplet Kit - 8 Runs (8x1)	3200616
Innovate Cartridges & Droplet Kit - 8 Runs (2x2 & 1x4)	3200617
Innovate Cartridge & Droplet Kit - 8 Runs (1x8)	3200618
Innovate Cartridges & Droplet Kit - 40 Runs (40x1)	3200619
Innovate Cartridges & Droplet Kit - 40 Runs (10x2 & 5x4)	3200620
Innovate Cartridges & Droplet Kit - 40 Runs (5x8)	3200621
Innovate Cartridge – 8 runs (8x1)	3200597
Innovate Cartridges - 8 Runs (2x2 & 1x4)	3200598
Innovate Cartridge - 8 Runs (1x8)	3200599
Innovate Cartridges - 40 Runs (40x1)	3200613
Innovate Cartridges - 40 Runs (10x2 & 5x4)	3200614
Innovate Cartridges - 40 Runs (5x8)	3200615