Encapsulation of Cells in Agarose on the Nadia Innovate and Nadia Instrument

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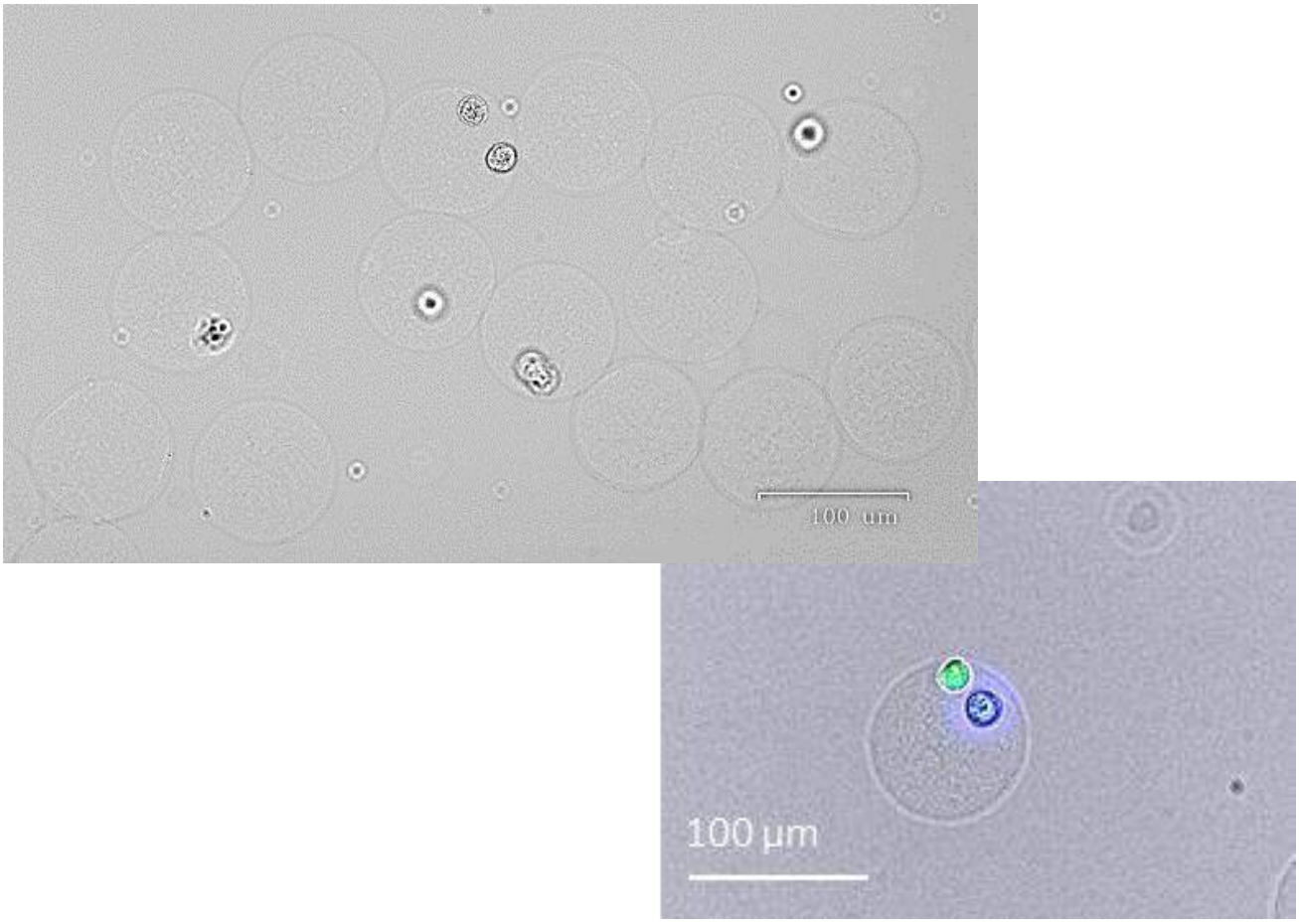

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Disclaimer

Summary

This application note describes the encapsulation of cells in agarose microdroplets. It details the steps taken to generate a custom protocol by changing parameters such as pressure, stirrer speed and temperature on the Nadia Innovate to encapsulate mammalian cells in 1 % agarose droplets (Figure 1). The subsequent recovery of the cell-containing agarose beads and culture of those cells within the 3D agarose scaffold is also explained in this document.

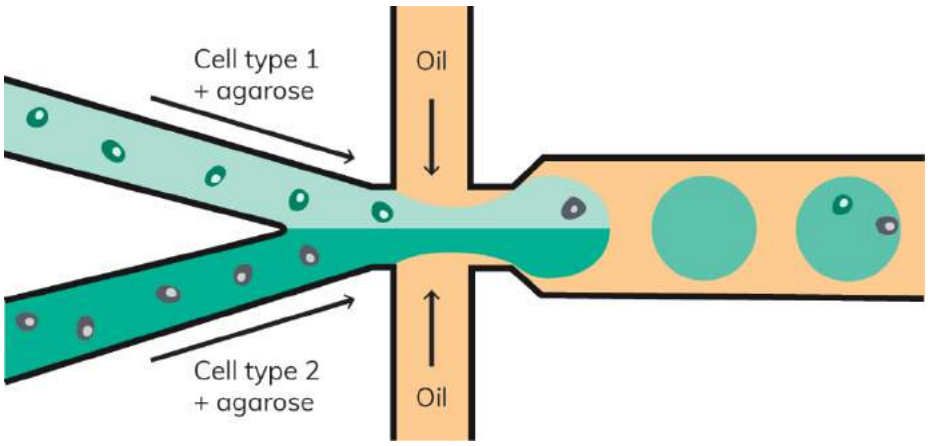

Figure 1: Schematic of cell-containing agarose droplet generation. Flow focussing allows droplets of agarose to be formed at high throughput. This technique was used in this application note to encase cells in the spherical scaffold provided by the agarose once it had hardened.

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 allows cells to be grown in individual microenvironments for extended periods of time (2).

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. This property of hydrogel beads can for instance be used in cancer research to study tumour development (3) and for drug screening (4) once colonies of cancer cells are established.

Growing cells in gel spheres 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 (5).

Furthermore, hydrogel bead-based cell delivery systems 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 with using droplet microfluidics, co-encapsulating living 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 (6). Developments in these areas are now increasingly possible due to the availability of microfluidic devices, such as the Nadia Instrument, which allow the co-encapsulation of two distinct cell types in agarose for high-throughput analysis of single cell-cell or cell-pathogen interactions over biologically relevant timeframes.

To this end, 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 describes the encapsulation and cultivation of mammalian cells (one or two cell types) in agarose beads using the Nadia Innovate, illustrating the instrument's potential in the field of single cell analysis in hydrogel scaffolds.

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 tested the encapsulation of cells in agarose and the recovery of the produced beads. Finally, we experimented with the cultivation of the encased cells over a period of 15 days.

Material and Methods

Droplet system. Dolomite Bio's Nadia Instrument 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. 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 (Figure 2), 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 Centre (FCC) software, allowing real-time observation and optimisation of droplet production via the Nadia Innovate high-speed digital microscope.

The Nadia platform uses disposable one-use plastic cyclic olefin copolymer (COC) chips to perform the encapsulation. This prevents reagent cross-contamination between runs and ensures solidified agarose within a cartridge does not affect subsequent runs. For the Nadia Innovate, single sample 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 1x PBS at a 2 % concentration and molten in a microwave oven. The agarose was filtered through a 0.2 µm syringe filter into a 14 ml Falcon tube which was stored in a beaker filled with water and placed on a hot plate set to 40 ˚C.

Optimisation of agarose encapsulation. The objective of this application note was to demonstrate the encapsulation of one as well as two cell types in agarose. To ensure that both cell types were subjected to the same treatment before encapsulation, the two cell suspensions were mixed with agarose before loading into the Nadia Innovate chip. For consistency throughout this work, the same principle was applied when encapsulating one cell type only, i.e. cells + agarose were loaded in the 'sample' well and 1x PBS + agarose were loaded in the 'bead' well (please note: the latter well is referred to as 'bead' reservoir due to previous nomenclature from RNA sequencing applications). A consequence of introducing agarose in both aqueous channels was the ability to achieve a final concentration of agarose inside droplets of 1 % by mixing a 2 % solution at a 1:1 ratio with cell suspension or 1x PBS prior to loading into the chip.

Prior to sample loading and throughout the duration of the run, the Temperature Control Unit (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 the Nadia Innovate platforms, 3 ml of QX200™ Droplet Generation Oil for EvaGreen (#1864005, BioRad) were loaded into the oil reservoir of the Nadia chip. 2 % agarose solution was mixed at a 1:1 ratio with pre-warmed (40 °C) 1x PBS and 250 µl of the mix were loaded in both the 'cell' and 'bead' wells. Encapsulation parameters, especially pressures, were optimised using the Nadia Innovate and Dolomite Bio FCC software with the aim to use up both aqueous solutions at the same rate. The effect of protocol changes on droplet generation was assessed in real time using the high-speed digital microscope camera video feed focussed on the microfluidic junction.

Cell preparation. Human Embryonic Kidney (HEK) 293 and mouse 3T3 cells were cultured in DMEM / 10 % FBS / 1x PenStrep until they reached 60-70 % confluency. On the day of each experiment, the culture media was removed and cells were washed with 10 ml sterile pre-warmed (37 °C) 1x PBS. TrypLE (3-5 ml for T25 and 5-10 ml for T75 culture flasks) was added and culture flasks were incubated at 37 °C for 3-5 mins to facilitate cell detachment. An equal volume of culture media was added to inactivate TrypLE and cells were collected in a 50 ml Falcon tube. Cells were centrifuged at 300 x g for 3 mins, the supernatant was removed and the cell pellet was re-suspended in 1 ml of sterile 1x PBS. Cells were centrifuged again, re-suspended in 1 ml of sterile 1x PBS and passed through 40 µm cell strainer. A 10 µl aliquot was taken, mixed with 10 µl of 0.4 % Trypan Blue stain solution and loaded into a Neubauer Improved haemocytometer to count the cells. The cell concentration was adjusted as needed with sterile 1x PBS.

Staining of cells. Cells were stained by adding either 20 µM calcein green or 1 mg / ml Hoechst 33342 stain solution at a 1:1000 or 1:5000 ratio to cell suspensions respectively and incubating for 10 mins on ice. After staining, cells were spun down 5 mins at 500 x g, the supernatant was removed, and cells were resuspended in fresh 1x PBS.

Encapsulation of cells in agarose beads. Using the optimised run parameters, cells were encapsulated in agarose beads using 3 ml of QX200™ Droplet Generation Oil for EvaGreen and 2 % agarose solution mixed at a 1:1 ratio with pre-warmed (40 °C) 1x PBS or cell suspension depending on whether one or two cell types were encapsulated during a particular run.

After a run had ended, the resultant emulsion was transferred from the Nadia chip output reservoir to a 1.5 ml microcentrifuge tube using a P1000 pipette. To harden the agarose beads inside the droplets, the emulsion was stored at 4 °C for 30 mins before being processed further. Emulsions and agarose beads were imaged using the Dolomite Bio high-speed digital microscope or a Zoe Fluorescence BioImager (BioRad).

Recovery of agarose beads. Agarose beads were recovered from the droplets via breaking of the emulsion with perfluorooctanol (PFO) (370533, Sigma-Aldrich) after collecting it in a 1.5 ml microcentrifuge tube.

Using a standard micropipette, 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 the side of the tube (around 300 µl of emulsion are produced per sample using the run parameters detailed in this application note). One volume of 1x PBS was added to one volume of emulsion to dilute the beads and make the aqueous phase easier to pipette after emulsion breakage. Two volumes of PFO were added to one volume of emulsion to break it and the tube was inverted five times by hand. The tube was then spun down for 30 s at 500 x g to separate the aqueous phase (containing the agarose beads) from the unwanted fluorous phase. Spinning down also helped to bring all agarose beads into the aqueous phase and stop them from sticking to the walls of the tube. After centrifugation, the tube was tilted at a 45-degree angle, and the aqueous supernatant layer was removed and transferred into a new microcentrifuge tube. 500 µl of 1x PBS were added to further dilute the agarose beads and make it possible to pipette the beads into a haemocytometer chip for further observation under the microscope.

Cultivation of cells in agarose beads. Following optimisation of the encapsulation parameters and the PFO recovery step, the developed method was used to encapsulate cells in agarose for subsequent 3D culture. This was done using a Nadia Instrument set up on a bench next to a cell culture hood.

To ensure that work was carried in aseptic conditions, sterile plasticware (e.g. pipette tips) was used and any non-sterile plasticware/equipment (e.g. tips boxes, micropipettes) and reagents (emulsion oil and PFO bottles) were thoroughly wiped with isopropanol (IPA). The 2 % agarose solution was filtered with sterile syringe and filter into a sterile 15 ml tube. A 2-way Nadia Innovate cartridge was taken out of its packaging, and the gasket as well as the bottom and sides on the cartridge were wiped with IPA inside the hood (note: Nadia chips themselves are made in a very clean, dust-free environment and therefore do not require cleaning before usage). Still to ensure aseptic conditions, 2x 3 ml of QX200™ Droplet Generation Oil for EvaGreen were loaded in the cartridge inside the hood (i.e. before the start of the run), the gasket was placed on top and the cartridge was carefully transported (to avoid spillages) to the Nadia Instrument. The run was started by placing the Nadia Innovate cartridge on the Nadia Instrument and choosing the relevant protocol. The Nadia progressed through the protocol steps until the user was prompted to load samples. At this point, the cartridge with the gasket on top was transported back to the hood, where pre-warmed 1x PBS or cell suspension was mixed with 2 % agarose solution and loaded into the chips. The cartridge and gasket were placed back onto the Nadia, which picked up the protocol where it was when the cartridge was temporarily removed and proceeded with encapsulation. Emulsion collection and agarose beads recovery were performed inside the hood using sterile consumables, equipment and reagents.

5 ml of DMEM / 10 % FBS / 1x PenStrep / 2.5 µg / ml Amphotericin B were added to T25 flasks and seeded with agarose beads equivalent to max. 160,000 cells to ensure media would not be depleted of nutrients before the end of the experiment. The inside and outside of a plastic box with hinged lid were wiped thoroughly with IPA and placed in a 37 °C incubator. Flasks were placed inside this quarantine box in the incubator to minimise the risk of contamination. The lid of the box was kept minimally ajar to ensure oxygen circulation.

To observe cells growing in agarose, 2-ml aliquots were taken from culture flasks at relevant time points, spun down for 2 min at 500 x g, the supernatant was removed, beads were re-suspended in 300 µl of 1x PBS and pipetted into a haemocytometer chip. Cells in agarose were observed at 0, 5, 10 and 15 days. Fresh media was added to the flasks after 7 days to make sure cells had enough nutrients available until the end of the experiment.

Optimisation of run parameters using the Nadia Innovate. To establish the dropletization protocol, sample loading, pre-run and run temperatures were set to 40 °C to ensure that the agarose solution remained molten for the duration of the run. Furthermore, the heat dissipation of the chip ensured that the temperature within the cell reservoir was maintained at approximately 37 °C. Stirrer speeds were set to 75 rpm for both 'cell' and 'bead' stirrers to avoid damaging cells when encapsulating two cell types at the same time.

To start with, pressures in the 'cell' and 'bead' lines were adjusted to ensure that the interface between both aqueous phases was in the middle of the microfluidic junction. This allowed confirmation that the two fluids contributed to 50 % of the volume of each droplet, a fact that has a knock-on effect on the required concentration of cells. Due to the asymmetry between the 'cell' and 'bead' channels, the pressure in the 'bead' line was lowered to 125 mbar and the pressure in the 'cell' line was increased to 210 mbar. At this stage of development, one of the two agarose solutions was dyed with Trypan Blue stain solution to visualize the interface (Figure 3, white arrows).

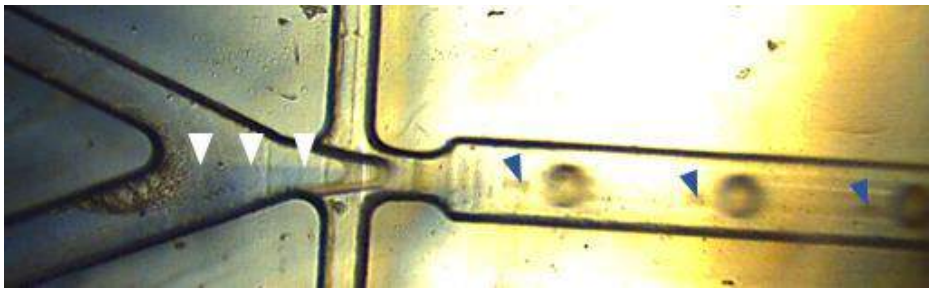

Figure 3: During optimisation of the run parameters, the agarose solution in the 'bead' line (bottom) was dyed with Trypan blue to visualize the interface between both aqueous phases (outlined with white arrows). Satellite droplets were also observed (shown with blue arrows).

As satellite droplets were observed with these conditions, the oil pressure was subsequently decreased from 450 to 400 mbar to reduce the size of those satellite droplets (Figure 3, blue arrows). Satellites are small droplets that can form just after a bigger droplet has been pinched off the aqueous stream by the oil phase. Viscous fluids such as agarose solution can be more prone to forming satellites. Such droplets are usually not considered a problem because they are too small to encapsulate a cell. However, during the optimisation of the protocol described in this application note, satellite droplets occasionally appeared to be almost big enough to contain a cell. Once the oil pressure was adjusted, no cells were visible in satellite droplets.

Pressures in the 'bead' and 'cell' lines were then fine-tuned to ensure that both fluids were consumed at the same rate. This led to the 'bead' pressure being lowered to 115 mbar. The priming steps leading to stable droplet formation were changed too, with pressures ramping up slowly in both aqueous channels to progressively bring agarose to the microfluidic junction. Finally, the length of the run using the optimised pressures was determined. To do this, the steady-state step in the pressure profile (Table 1) was set to 40 mins and the resulting protocol was used to run a Nadia Innovate chip loaded with maximum volume of reagents (3 ml for oil, 250 µl for aqueous solutions) until one of the reagents ran out. After just under 15 mins, the 3 ml of oil ran out. Within the same amount of time, 150 out of 250 µl of aqueous solutions were processed. To avoid irregular droplet formation towards the end of the run, it is important to ensure that no line runs dry, i.e. that a small amount of liquid is left behind in each well. To comply with this requirement, the steady-state step was set to 14 mins 40 s and the sample/agarose volume was set to 200 µl (Figure 4).

Table 1: Pressure profile for generating 1 % agarose droplets with the Nadia Innovate and Nadia instruments using 1 % agarose solution in both aqueous channels. The steady-state step, during which most droplets are produced, is outlined with a double line. Priming steps, encapsulation steps and post-run steps are highlighted in blue, green and grey respectively.

Step #	Step Duration (hh:mm:ss)	Oil Pressure (mbar)	Agarose 1 (Bead) pressure (mbar)	Agarose 2 (Cell) pressure (mbar)
1	00:00:01	400	0	0
2	00:00:05	400	90	90
3	00:00:05	400	100	100
4	00:00:05	400	105	150
5	00:00:05	400	110	200
6	00:14:40	400	115	210
7	00:00:01	400	115	210
8	00:00:01	400	0	0
9	00:00:01	0	0	0

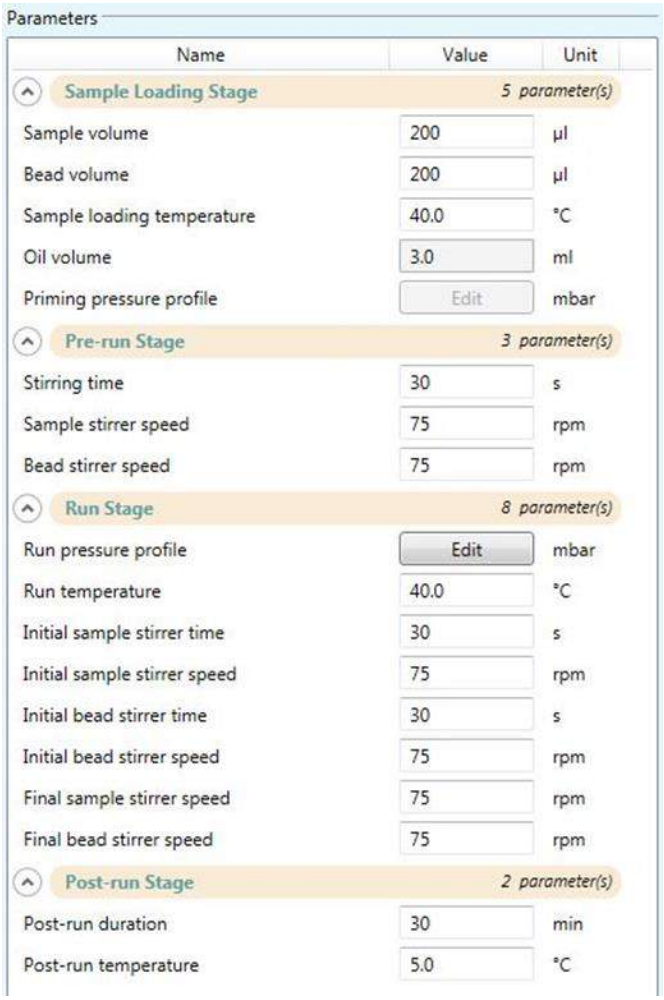

Figure 4: Summary of editable parameters used in this application note taken from the Dolomite Bio FCC software.

The optimised Nadia Innovate protocol enabled the production of monodisperse droplets (Figure 5, A) and, after PFO extraction, semi-transparent agarose beads with an average diameter of 85 µm (Figure 5, B).

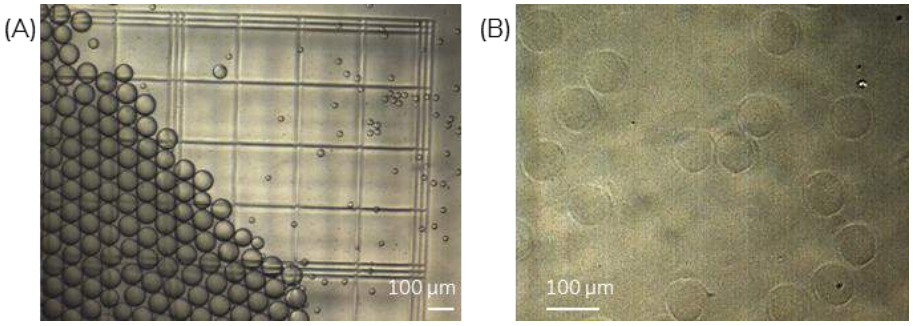

Figure 5: (A) Agarose emulsion produced with the optimised Nadia Innovate protocol, showing good monodispersity and the presence of a small number of small satellites alongside the droplets of interest. (B) 85 µm agarose beads after PFO extraction.

Encapsulation of cells in agarose beads. Using the protocol described in the previous section, Hoechst-stained HEK cells were encapsulated in agarose beads. Cells were prepared at a concentration ensuring that 1 in 5 droplets contained a cell (Figure 6, A and B). This was calculated the following way:

- Droplets were 85 µm in diameter, which equated to a volume of 322 pl.
- We wanted 1 cell in 5 droplets and the cell suspension contributed to 50 % of the volume of each droplet. We therefore wanted 1 cell in 5 x (322 / 2) = 805 pl.
- This equated to 1242 cells in 1 µl, or 1.2 M cells in 1 ml.
- Given that cells were mixed with agarose at a 1:1 ratio before loading into the Nadia Innovate chip, cells were prepared at a concentration of 2 x 1.2 = 2.4 M / ml.

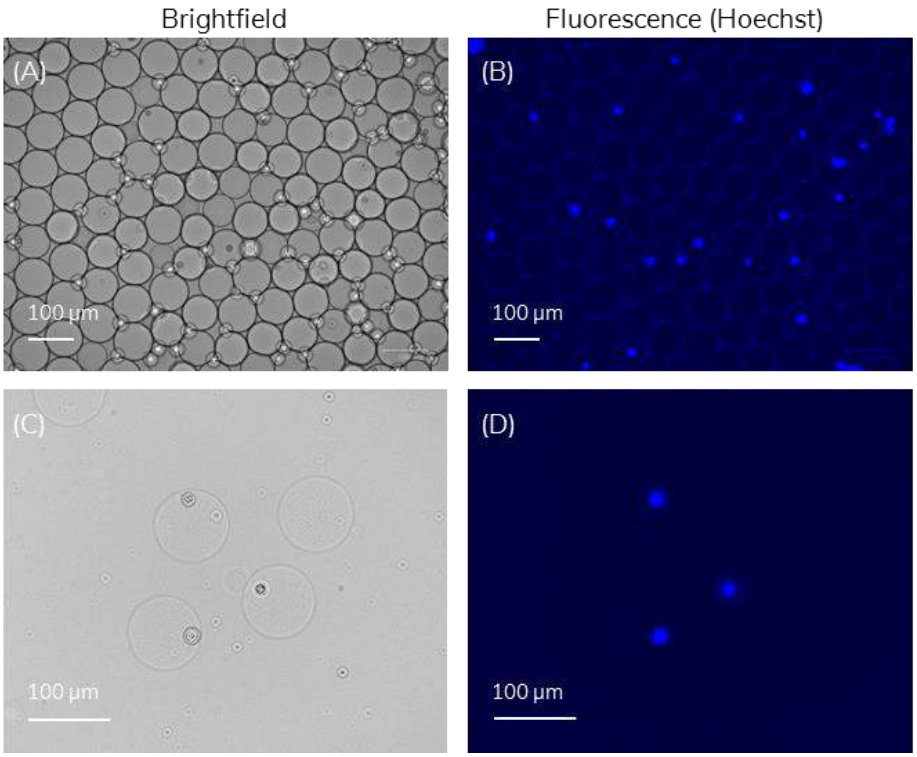

Figure 6: (A) and (B) Hoechst-stained HEK cells were successfully encapsulated at a distribution of 1 cell per 5 droplets and no cells were visible in satellite droplets. (C) and (D) Agarose beads containing HEK cells were successfully extracted from the emulsion. Fluorescence indicated that cells were alive and did not suffer from being briefly exposed to PFO.

Following encapsulation and PFO extraction, fluorescent HEK cells were observed in the generated agarose droplets (Figure 6, C and D). The fact that cells were fluorescent indicated that living cells were not adversely affected by being briefly exposed to PFO.

To demonstrate co-encapsulation of cells, Hoechst-stained HEK cells and Calcein-stained 3T3 cells were encapsulated together. Both cell types were prepared at a concentration of 2.4 M / ml to ensure that 1 in 5 droplets contained either cell type. Based on this theoretical distribution, the expected frequency of droplets containing two cells was 1 / 5 x 1 / 5 = 1 in 25 droplets. Living, fluorescent cells were observed in the produced droplets and in the extracted agarose beads (Figure 7). Co-encapsulated HEK and 3T3 cells were detected at the expected frequency.

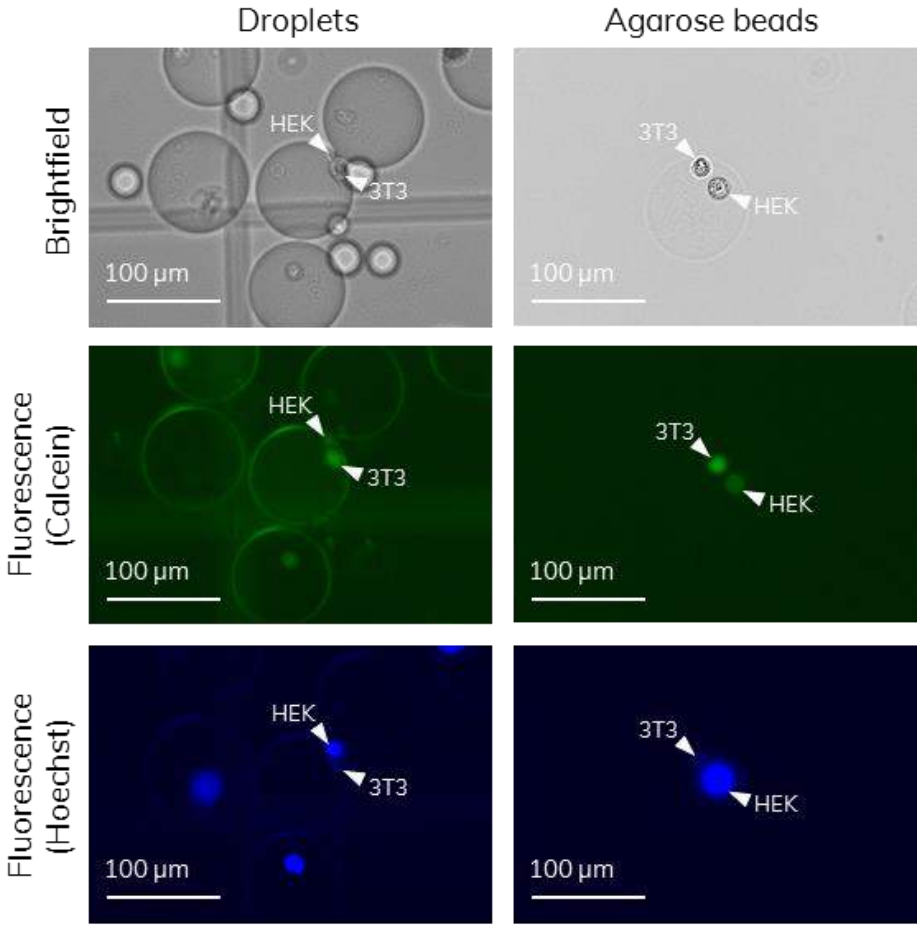

Figure 7: Hoechst-stained HEK cells and Calcein-stained 3T3 cells were encapsulated together. Living, differentially-stained, fluorescent cells were observed in both the produced droplets and the extracted agarose beads.

3D cell culture in agarose beads. Once encapsulated, cells were incubated at 37 °C for 15 days in presence of culture media. Living cells were observed at all timepoints, confirming that the necessary nutrients and oxygen were freely available to them while inside the agarose (Figure 8). Cell growth was relatively marginal within the timeframe of the experiment. This observation calls for further optimisation of the 3D culture conditions, such as adjusting the agarose concentration or increasing nutrient flow. The mesh formed inside the 1 % agarose beads as they hardened may indeed have been too tight and partially inhibited cell growth, warranting further exploration using less dense hydrogels. However, culture optimization was outside the scope of this application note.

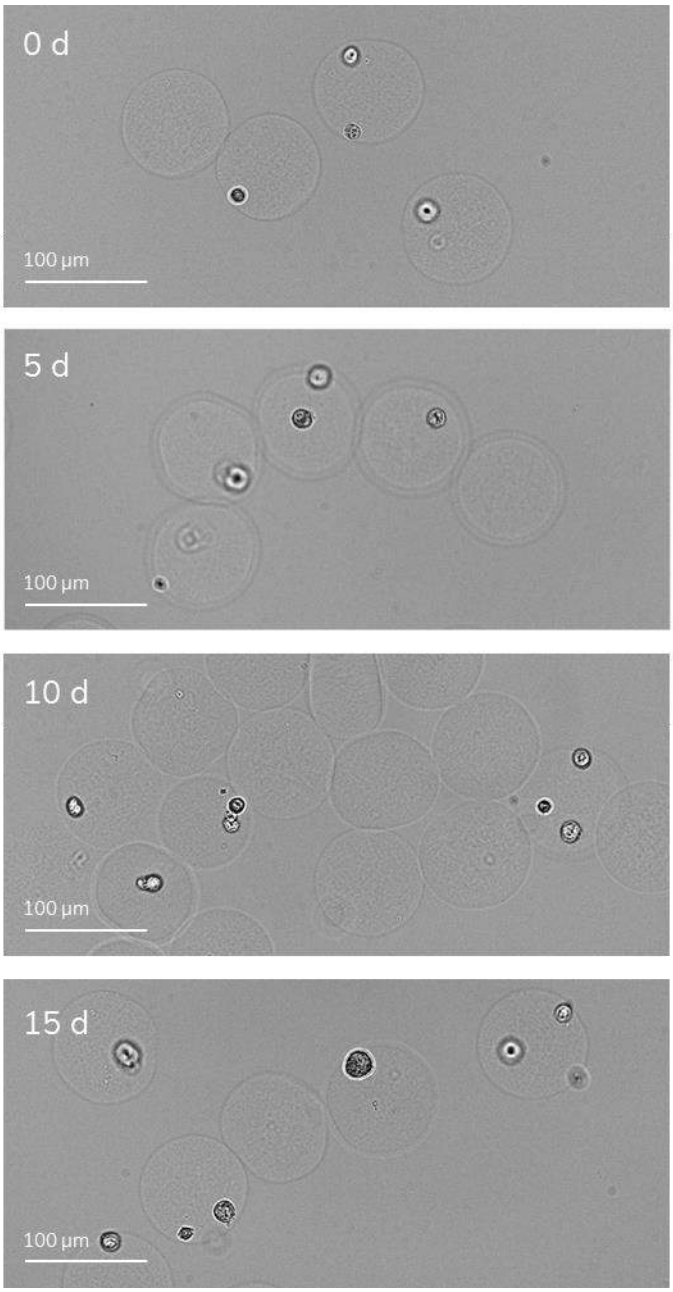

Figure 8: Living HEK and 3T3 cells showed marginal growth within the 3D scaffold provided by the agarose over a period of 15 days.

Conclusion

Hydrogel droplet encapsulation is a useful tool for the individual culturing or imaging of singulated cells. This application note showed that by using the Nadia Innovate system and the Nadia instrument, live cells could be readily encapsulated with agarose inside droplets and solid agarose beads could be recovered. Cell-containing agarose beads were then re-suspended in culture media and incubated at 37 °C to allow for cell growth in a 3D microenvironment. These experiments highlighted the suitability of the Nadia product family for hydrogel bead-based applications in 3D single-cell research.

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Product Information

Nadia Innovate system

Description	Part number
Nadia Instrument	3200590
Nadia Innovate	3200595
Nadia Innovate Upgrade for Modular Systems	3200660

Nadia Innovate consumables

Description	Part 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