**3-Component Bead Formation**

**Creating Solution**

1. Check cell confluency
	1. If cells cover entire flask bottom (confluent), passage cells, taking a third of the cells for bead making
		1. To passage cells, first suck out media from the culture flask, then wash cells with 3mL of PBS
		2. After sucking out the PBS, put in 1mL trypsin to allow the cells to detach from the flask surface
		3. Suck out the trypsin immediately, as prolonged exposure to trypsin may damage cells
		4. Put the flask back in the incubator for around 2 minutes
		5. Check to see if cells have detached from the surface by hitting the sides of the flask and holding it at an angle; if cells begin to slide then they are detached
		6. Add 6mL media, pipette up and down to suspend the cells evenly
		7. Transfer 2mL of the pipetted solution into a 15mL Falcon tube
		8. Add 3mL of media and centrifuge at 1000rpm and 22 degrees for 5 minutes
		9. Suck out media
2. Create shell solution
	1. Transfer 1mL of alginate into a 1.5mL Eppendorf tube
	2. Pipette the CaCO3 solution up and down until homogeneous, take from bottom, release on top
	3. Add 40μL of CaCO3 into the Eppendorf tube to make 80mM CaCO3
	4. Vortex mix the tube until homogeneous
3. Create core solution
	1. Take out collagen and matrigel, ensuring they are kept in a small box of ice the entire time
		1. Both gel extremely quickly when removed from a cold environment
	2. Put several 200μL pipette tips and an Eppendorf tube into the ice to prevent the collagen or matrigel from heating
	3. Collagen, matrigel and alginate are all quite viscous, so use a precise pipette (the grey and yellow one)
	4. Transfer 55μL (set the pipette to 65 as it is very viscous) of collagen into the Eppendorf tube
	5. Transfer 100μL (105 as it is very viscous) of matrigel into the Eppendorf tube
	6. Add 3μL of NaHCO3 to the Eppendorf tube adjust the pH to desired value
	7. Transfer 150μL (160) of alginate into the Falcon tube with the cells, pipette up and down to suspend evenly
		1. Avoid bubbles by pipetting gently and keeping the tip inside the liquid
	8. Add the alginate and cell mix to the collagen matrigel mix
	9. Hold the tube at an angle and flick it vigorously to make the solution homogeneous – this takes a little while

**Making Mineral Oil Solutions**

1. With SPAN80 (Inlet 1)
	1. Fill designated 50 mL Falcon tube with 40 mL of mineral oil
	2. Add 0.5 g of SPAN80 to the tube
	3. Fill with mineral oil until the 50 mL line
	4. Add 50 μL of acetic acid
2. Without SPAN80 (Inlet 4)
3. Fill designated 50 mL Falcon tube with 50 mL of mineral oil
4. Add 50 μL of acetic acid

**Bead Creation**

1. Set up Fluigent
	1. Connect the Fluigent to the power source and the computer via USB.
	2. Open the gas valve (grey switch should be parallel to the tubing)
	3. Turn on the Fluigent (turn on the round black switch on the left side of the back of the machine, then press the green button on the left)
		1. **Ensure that the pressure reading on the machine never exceeds 1000 mbar. Exceeding 1034 mbar will damage the expensive machine.**
	4. Log in to the computer’s Admin account. The password is, as of 26 June 2014: Flui@)13Gent!
	5. Turn on “MAESFLO 3.1.3”. Click “OK” and “SKIP” for pop-ups.
	6. **Ensure that there is never any liquid in tubes connected directly to the Fluigent. This will damage the machine.**
2. Place a bead forming chip on a petri dish, and check it under the microscope to make sure there are no blockages
3. Fill the cooling system with ice and water (to keep the core cool)
	1. Make sure the red bucket used for collecting water will not overflow
	2. Use the tube that is embedded within a thicker tube that can be connected to the cooling system
4. Set up the chip
	1. The first inlet should flow mineral oil with acetic acid and span
	2. The second inlet should flow the shell solution
	3. The third inlet should flow the core solution (tubing should be connected to the cooling system
	4. The fourth inlet should flow mineral oil with only acetic acid
	5. The remaining channel is used for collection, where an exit tube should be connected
		1. Connect the exit tubing into a 50mL Falcon tube filled with media warmed to 37 degrees for collection
5. Flow the solutions in this order
	1. The first at around 100mbar
	2. The fourth at around 100mbar
	3. The second at around 100mbar (200 or more at first if very viscous)
	4. The third at around 100mbar (200 or more at first if very viscous)
6. Pressures can be adjusted
	1. If the cell solution is not pinching and flows as a stream, increase channel 1 and 4 pressures or lower channel 3 pressure
	2. If the cell solution is not flowing, increase channel 3 pressure or decrease 1 and 4
	3. To increase or decrease bead formation rate, increase or decrease channel 3 pressure, respectively
7. Switch media every 30-40 minutes, as cells may be damaged from prolonged exposure to the acetic acid
	1. Suck out the oil layer from the top of the liquid
	2. Use a 10mL pipette and transfer 13mL of the bead-filled media into two 15mL Falcon tubes
	3. Centrifuge at 1000rpm and 22 degrees for 5 minutes
	4. Suck out media, then suspend each tube’s beads in 2mL complete media
	5. Pipette up and down to make sure all beads are collected
	6. Transfer the beads into a flask, and fill the flask with an extra 6mL of complete media
	7. Label the flask with details of the bead (amount of each reagent, cell line, etc.), the date, the batch number, and your name

**Clean-up**

1. Fluigent
	1. Remove tubes from the Fluigent.
	2. Turn off the Fluigent (press the red button, and then the black switch on the left side of the back of the machine)
	3. Close the gas valve (grey switch should be perpendicular to the tubing)
	4. **Keeping either Fluigent or gas on for extended periods will damage the machine.**
2. Run all tubing with 70% ethanol
3. Wipe the microscope glass
4. Detach the cooling system
5. Wipe any oily surfaces