LAB PROCEDURES

STAIN

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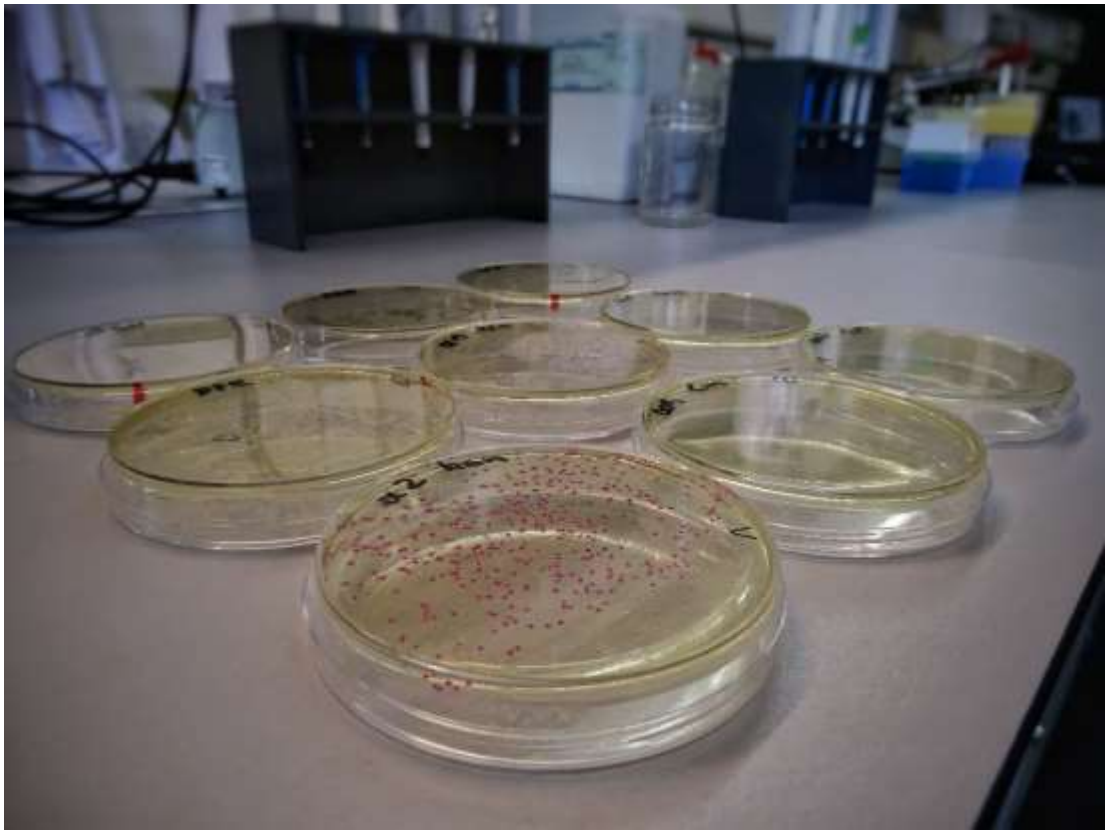
1. SYNTHETIC BIOLOGY PROTOCOLS



A. GENE ASSEMBLY PROTOCOL

- Gene assembly means to join different DNA sequences or genes into a single plasmid backbone also called vector. The vector is a vehicle that is used to maintain a "genes of interest" or a "transcriptional unit" in an individual autonomous replicative genetic element.
- The process of the assembly makes use of restriction endonucleases that are used to cut DNA (genes of interest and vector) into DNA fragments with defined compatible ends that then can be re-joined at will with T4 ligase enzyme that glues all fragment together within a vector backbone.

We use the Golden Gate Assembly/ Golden Braid Assembly That allows simultaneous gene assembly of different DNA sequences by the action of a Type II restriction endonuclease and a ligase within a single reaction.

MATERIALS:

EQUIPMENT:

- Pipet and pipette tip
- Eppendorf tubes (0,2 ml)
- Thermocycler

CONSUMABLES:

- Sterilized water (Milli-Q)
- Ligase mix (NEB) (buffer 10x)
- Master mix (new England Biolabs)

Assembly Level 1

- pARK_Alpha1 (Vα1)
- pARK_Aalpha2 (Vα2)
- Promoter (Part:BBa_K2656004)
- RBS (Part:BBa_K2656009)
- *Camelia sinensis* flavanone3-hydroxylase (F3H)
- *Fragaria ananassa* dihydroflavonol 4-reductase (DFR)
- *Petunia hybrida* anthocyanidin synthase (ANS)
- *Arabidopsis thaliana* anthiocyanin 3-*O*-glucosylransferase (3GT)
- TERMINATOR (Part:BBa_K2656026)

Assembly level 2

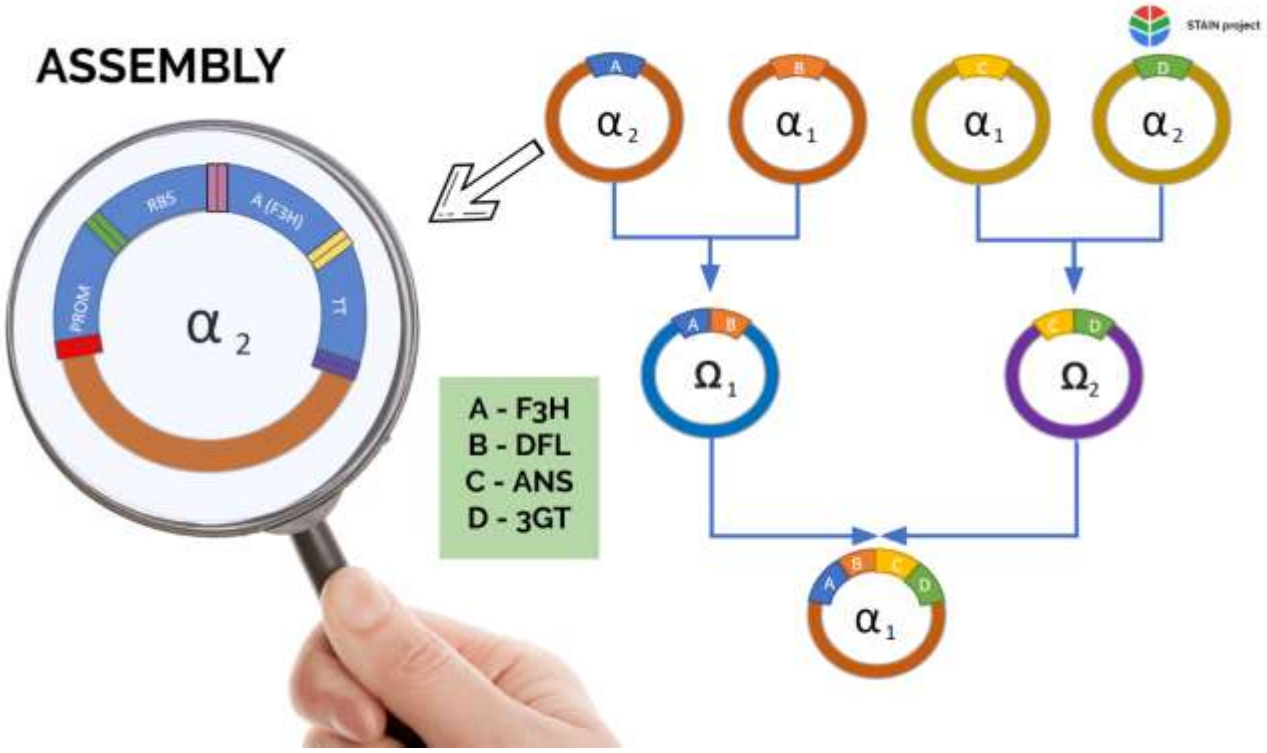
- pDGB1_Omega 1 (VΩ1)
- pDGB1_Omega 2 (VΩ2)

PROCEDURE: Pipetting

	TIME	SPEED	VOLUME/MASS
MEASURE PLASMID´S CONCENTRATIONS			
DNA OF EACH PLASMID/PART NEEDED			75 ng
ADD IT TO A BRAND NEW 200 µl TUBE			
ADD MASTER MIX			1 µL
ADD LIGASE MIX			2 µL
ADD MILLI-Q WATER UNTIL 20µL			

PROCEDURE: Assembly

TAKE THE TUBE AND INTRODUCE IT IN THE THERMOCYCLER			
THERMOCYCLER	STEPS TO ENTER INTO THE 200µL THERMOCYCLER		
	NUMBER OF CYCLES	TEMPERATURE	TIME
	1 CYCLE	37ºC	10 MIN
	27 CYCLES	• 37ºC ----------1´30" • 16ºC ----------3 MIN	
	1 CYCLE	50ºC	5 MIN
	1 CYCLE	80ºC	10 MIN



ASSEMBLY LEVEL 1

α-1 vector

A

P007	RBS 009	CDS 3GT	TERM - PI	V α1

B

P007	RBS 009	CDS ANS	TERM - PI	V α1

C

P007	RBS 009	CDS F3H	TERM - PI	V α1

D

P007	RBS 009	CDS DFR	TERM - PI	V α1

α-2 vector

A

P007	RBS 009	CDS 3GT	TERM - PI	V α2

B

P007	RBS 009	CDS ANS	TERM - PI	V α2

C

P007	RBS 009	CDS F3H	TERM - PI	V α2

D

P007	RBS 009	CDS DFR	TERM - PI	V α2

ASSEMBLY LEVEL 2

Ω-1 vector

Transcriptional unit **A** (from α1)	Transcriptional unit **B** (from α2)	Vector backbone **Ω1**

Ω-2 vector

Transcriptional unit **C** (from α1)	Transcriptional unit **D** (from α2)	Vector backbone **Ω2**

ASSEMBLY LEVEL 3 (final assembly)

α-1 vector

Transcriptional unit A+B (from Ω1)	Transcriptional unit C +D (from Ω2)	Vector backbone **α1**

B. TRANSFORMING BACTERIA

- Transforming bacteria basically means to transfer the assembled plasmids inside the bacteria (*E. coli* in our case), by a procedure which involves an extreme change of temperature or "heat-shock", to force the bacteria to adsorb the plasmid.
- This process must be done always carefully done with great precision as exact timing is important for successful transformation.

In this protocol, the use of competent bacterial cells is mandatory. Competent cells are cells that are grown in Luria Bertani broth (LB; 10 g Tryptone, 5 g Yeast extract, 10 g NaCl) to an optical density of 0.2. Cells are centrifuged and pellet is resuspended in 0.1 M $CaCl_2$ and incubated for 30 min at 4ºC. These cells could either be flash-frozen and stored at -80ºC or directly used to transform plasmid of interest. **Note:** BE CAREFUL TO NOT FREEZE CELLS ONCE THAWED FOR USE AS THEY LOOSE COMPETENCE

MATERIALS:

EQUIPMENT:

- Pipet and pipette tips
- A cube with ice
- Thermoblock
- Sterilized area.

CONSUMABLES:

- Bacteria culture media (LB)
- Competent cells
- 20µL of assembled plasmid

PROCEDURE:

	TIME	SPEED	VOLUME	TEMPERATURE	CONDITIONS
PREPARE YOUR 50µL TUBE OF COMPETENT CELLS					
ADD ASSEMBLED PLASMID			20µL		STERILIZED AREA
TAKE IT INTO ICE	30´				
THERMOBLOCK	2´30" EXACTLY!!!			42ºC	
BACK TO ICE	5-10 MIN				
ADD CULTURE MEDIA			1mL		STERILIZED AREA
THERMOBLOCK	1 HOUR	300 RPM		37ºC	

C. PLASMID MINIPREP PROTOCOL

➤ Miniprep is a process used for lysing bacteria cells in order to release the plasmid DNA.

We used the Nucleospin ▫ Plasmid Transfection-grade kit (740490.50 / Macherey-Nagel). To increase the yield, we performed a few modifications of the standard protocol (marked in red)

MATERIALS:

EQUIPMENT

- Thermoblock / Water bath
- Centrifuge
- Freezer
- Vortex
- Pipette and pipette tips
- Eppendorf tubes (1,5 mL)
- Plasmid TG column

CONSUMABLES

- Transformed *E. coli (*strain DH5ᾳ)
- Sterilized water
- BUFFER A1
- BUFFER A2
- BUFFER A3
- BUFFER ERB
- BUFFER AQ
- BUFFER AE
- Nucleospin ▫ Plasmid Transfection-grade kit (740490.50 / Macherey-Nagel)

PROTOCOL:

	TIME	SPEED	VOLUME	TEMPERATURE
CENTRIFUGE CULTURE	30''	11000 G		
REMOVE SUPERNATANT				
FREEZE PELLET	10'			
ADD BUFFER A1			250 µL	
VORTEX				
BUFFER A2			250 µL	
MIX INVERTING TUBE. 6-8 TIMES				
BUFFER A3			300 µL	
MIX INVERTING TUBE. 6-8 TIMES				
CENTRIFUGE	10'	FULL SPEED		
NEW EPPENDORF WITH PLASMID TG COLUMN				

ADD SUPERNATANT TO THE COLUMN				
CENTRIFUGE	1’	L		
REMOVE RESULTANT LIQUID				
ADD BUFFER ERB (1º WASH)			700 µL	
CENTRIFUGE	1’	11000 G		
ADD BUFFER AQ (2º WASH)			650 µL	
CENTRIFUGE	1’	11000 G		
REMOVE RESULTANT LIQUID				
CENTRIFUGE (DRY)	1’	11000 G		
COLUMN IN NEW EPPENDORF				
THERMOBLOCK	2’			70 º C
HEAT BUFFER AE				50 º C
ADD BUFFER AE			50 µL	
INCUBATE	1’			RT
CENTRIFUGE	1’	11000 G		
ADD RESULTANT LIQUID TO THE COLUMN AGAIN				
CENTRIFUGE	1’	11000 G		

D. AGAROSE GEL - ELECTROPHORESIS

➤ In order to know the size of the plasmid you work with, an electrophoresis can be done. The equipment has two zones with different electric charges which attract and separate the elements depending on their size and electric charge. This DNA samples are run through an agarose gel that is placed inside the electrophoresis machine.

MATERIALS:

EQUIPMENT

- Flask
- Microwave
- Electrophoresis equipment (machine)

CONSUMIBLES

- Agarose (powder)
- TAE buffer
- loading buffer 6x
- Molecular-weight size marker
- Plasmid sample (ANS and F3H genes)
- Cut plasmid samples (ANS and F3H genes)
- GEL RED
- NaCl

PROCEDURE FOR AGAROSE GEL:

	TIME	SPEED	VOLUME/ MASS	TEMPERATURE
PLACE AGAROSE POWDER			0,42g	
ADD TAE BUFFER			260mL	
TAKE IT INTO THE MICROWAVE UNTIL HOMOGENEOUS				
PLACE IT INTO AN ELECTROPHORESIS CONTAINER AND LET DRY OUT USE IT WHEN IT HAS A JELLY TEXTURE				

PROCEDURE FOR ELECTROPHORESIS:

	TIME	SPEED	VOLUME	TEMPERATURE	CONDITIONS
ADD SAMPLE FOR THE MACHINE			50µ		
PREPRING THE GEL RED					
ADD GEL RED			45µL		
ADD NaCl (O,5M)			30µL		
ADD WATER			117µL		
PLACE THE GEL INTO THE ELECTRPHORESIS CUVETTE					
(REMOVE THE COMB) PIPETTE THE MOLECULAR SIZE MARKER INTO 1 WELL AND SAMPLES INTO THE REMAINING WELLS					
SET THE MACHINE TO 80-100 V					
RUN IT UNTIL SAMPLES GET TO THE END OF THE GEL					
TAKE GEL OUT, LOOK IT A UV LIGHT TO SEE RESULTS					

2. WORKING WITH NATURAL MATERIALS

A. PROTOCOLS FOR PIGMENTS EXTRACTION

- The first step of the project is to extract the colour from the materials used (fruits/vegetables).
- The inks that are obtained from these extractions can be used later in order to do testing or create a product.

MATERIALS:

EQUIPMENT
- Smashing equipment

CONSUMIBLES
- Fruit / Vegetables / Organic material which used to extract the pigment

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PROCEDURE :

1. Take the sample into a mortar / beaker
2. Add some water
3. Smash the sample:
   (AMARANTH: freeze it in liquid nitrogen before smashing it)

   (RED CABBAGE : heating it up makes the smashing much simpler)
   (Also, the use of a potter-elvehjem makes the extraction easier)

   (SPINACH : better results using acetone instead of water)

4. Take the resultant liquid using a filter paper funnel if necessary

B. YEAST CELL FREE SYSTEM

- This experiment consists on producing a cell free system that stops the natural oxidation of the inks by reducing them at the same time that makes the colour of the inks more stable.

MATERIALS:

EQUIPMENT

- Mortar
- Scale (microbalance) (lab balance)

CONSUMIBLES

- fresh yeast
- Water
- Sand
- Silica Gel
- Inks

PROCEDURE:

1. Weight 6,5 g of Yeast (*Saccharomyces cerevisiae*) .
2. Smash it on the mortar.
3. Add 3,5g of water.
4. Smash on the mortar.
5. Add 1.5g of silica gel
6. Add 0,15g of sand.
7. Smash on the mortar.
8. The result must be a liquid cell free system made by the components of the cells of the previous yeast.

   **For our experiment the prepared yeast was centrifuged, and the pellet was removed and discarded. (The supernatant was enough for us to stop the oxidation).

C. CHARACTERIZATION

i. CHARACTERIZING INKS ON THE SPECTROPHOTOMETER

> The spectrophotometer allows to characterize the different colours from the extractions by relating the absorbance of the extracted material with their wavelength. This data is key to monitor compound oxidation and to visualize its effect on each colour.

MATERIALS:

EQUIPMENT:
- Spectrophotometer
- Pasteur pipette
- Cuvette

CONSUMIBLES:
- Sample
- MilliQ water

PROTOCOL:

1) Dilute the sample until the absorbance is below three.
2) Set the parameters: wavelength between 300 and 750.
3) Measure the reference. It depends on the solvent of the dilution, in this case is deionized water.
4) Put the sample in the cuvette with a Pasteur pipette.
5) Introduce the cuvette in the spectrophotometer.
6) Start the measure.

To see an example, here we have a graphic that sum up the oxidation of the spinach:

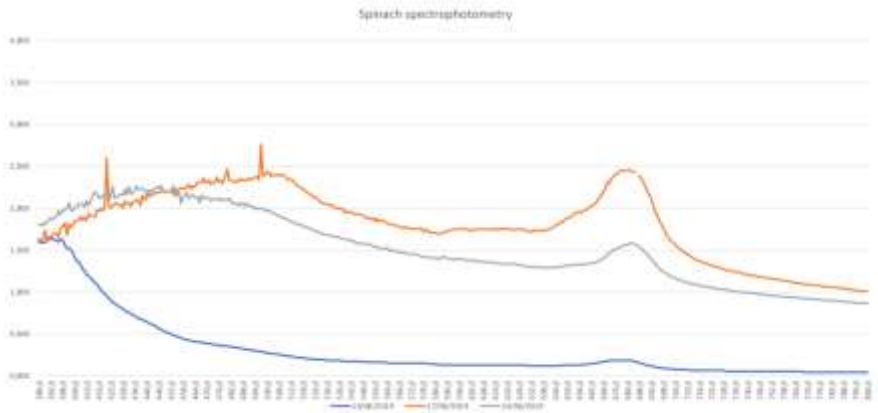


Figure 1. An example of a measure with the spectrophotometer.

In some graphics there are two or even three peaks. This shows that there are some inks that have different pigments which give different colours to the ink.

ii. CHARACTERIZING INK PIGMENTS BY TLC

- Another way to show what the inks or extracted pigments are made of, working with Thin Layer Chromatography (TLC) helps to separate and see the colours that make up the pigment. By placing a filter paper inside a $CaCl_2$ solution with some drops of the pigment, the colour disgregation can be done easily.

MATERIALS:

EQUIPMENT

- 250 mL Beaker
- Pipettes and pipette tips
- Pencil

CONSUMIBLES

- filter paper
- water
- CaCl2
- Extracted pigments (Inks)

PROCEDURE:

1. Cut a small rectangle of filter paper
2. Make (1cm) marks on the side of the paper (with a pencil) to place the ink.
3. Put 5µL of ink on the marks using a pipette.
4. Let the inks to dry
5. Put 50 mL of water on a beaker.
6. Add the CaCl2 making a solution.
7. Place the paper in the beaker with the ink at the bottom part.
8. The ink must start running up separating the different components.

D. CHANGES IN RED CABBAGE INK

➤ In order to obtain different colours from the cabbage ink, pH changes can be done. Introducing different buffer solutions into the inks make big visual changes on the colour of the organic liquid. Instead of working with a great variety of vegetables, changing the pH of the red cabbage is a better option, but it is also more dangerous to human consumption.

MATERIALS:

CONSUMIBLES

- Red cabbage extract
- Citric acid
- disodium phosphate
- Sterile 15mL tube (Falcon)

PROCEDURE :

1. Prepare the buffer solutions with the following proportions:

pH	Na_2HPO_4 (0.2 M) mL	Citric Acid (0.1 M) mL
2.2	0.40	19.60
3.2	04.94	15.60
4.2	08.28	11.72
5.2	10.72	09.28
6.2	13.22	06.78
7.2	17.39	02.61
8	19.45	00.55

2. Prepare each pH solution with the following proportions:

OPTIONS	RED CABBAGE EXTRACT(%)	BUFFER (%)
1	10	90
2	50	50

E. OTHER STUDIES

i. LYOPHILIZATION

➤ Another way to keep colour in a good condition is to lyophilize it. The process means introducing the sample inside a vacuum chamber machine that absorbs all the water, leaving a coloured powder that can be used later.

MATERIALS:

EQUIPMENT

- Lyophilizer

CONSUMIBLES

- 1.5- or 2-mL Eppendorf tubes
- Parafilm
- Color extracts
- Wooden toothpick

PROCEDURE:

1. Place the inks to lyophilize on the tubes.
2. Cut the lid
3. Cover the mouth of the tube with 3 layers of parafilm.
4. Make four holes with the wooden toothpick.
5. **Place the tubes on the jars of the lyophilizer.
6. Lyophilize the samples.

***** The lyophilizer must be prepared hours before the process is done with the pertinent pre-sets.
** It is useful to use paper to ensure they don't move during the process.

ii. STUDYING JUICE VISCOSITY

- To ensure that the ink texture is appropriate to use it on a pen or in a printer, its viscosity must be studied.
- To study this property, a solid sphere is passed through the liquid substance in order to obtain the data that we need to calculate the viscosity of the fluid.

MATERIALS:

EQUIPMENT
- Test tube
- Marble
- Ruler

CONSUMIBLES
- Strawberry juice

PROCEDURE :

1. Fill the test tube with the juice studied.
2. Measure the distance (cm) between two different lines of the test tube.
3. Drop the marble and measure the time the marbles takes to pass the previous marks.
4. Measure the diameter of the marble.
5. Calculate the marble density.
6. Calculate the density of the fluid.
7. With the data obtained in step nº 3, calculate the speed of the marble.
8. With all the previous data, calculate the viscosity of the strawberry juice with this formula:

Ps= Sphere density
Pf= fluid density
Vs= Volume of the sphere
η= viscosity
r= ratio of the sphere

$$\eta = \frac{4}{9} \cdot r2 \quad \frac{r2\, g\, (Ps - Pf)}{Vs}$$

3. MICROFLUIDICS

A. CREATING GLASS-CAPILLARY MICROFLUIDIC CHIPS

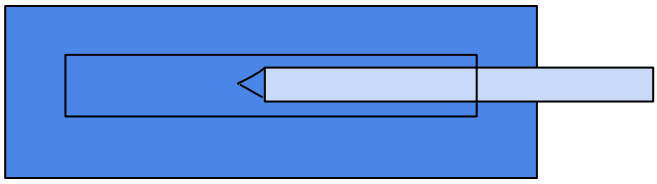
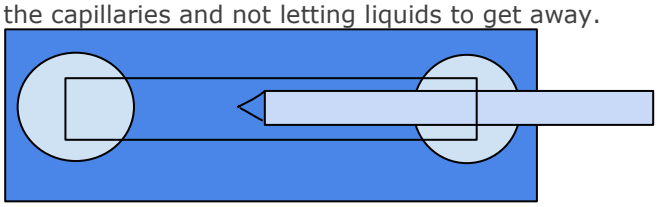
- The aim of this experiment is to create a device that introduces the ink inside a sphere (encapsulation) of another material, protecting the inks from oxygen and oxidation.
- There are a lot of different chips that can be create. In this experiment the device that is needed is the one that introduces two liquids through different syringes. This process creates a stable microfluidic production by adjusting the quantities and the timing so that lots of spheres are produce per second.

MATERIALS:

EQUIPMENT:

- 2 syringe tips
- Epoxy resin
- 2 capillaries of 15cm, cut to 4,5 cm. (one is squared, the other one has a sharp end)
- A microscope slides
- Gloves

BUILDING THE CHIP:

1. After getting all the materials ready, imagine the chip we want to create, there will be two entries for liquids and one exit for bubbles. So, first, place the plate.
2. Take the sharp ended capillary and introduce the sharp end inside the squared-ended capillary, more or less till the middle of it.
3. Stick using Epoxy the squared capillary making the sharp-ended capillary´s other end stand out of the plate.
4. 
5. **Be careful with Epoxy resin, it is very dangerous for the skin.**
6. Then it is time to stick the syringes, one at each of the ends of the squared-ended capillary. And glue it very good, make little incisions in the bottom of the syringe tip in order to fit perfectly with the capillaries and not letting liquids to get away.
7. 
8. Be sure to glue everything in the right way and without leaving any exists for colour.

B. HOW TO CREATE MICROFLUIDIC SPHERES

- The aim of the experiment is to stop natural oxidation by enclosing the natural pigments inside substances that isolate them from O2 action.
- In this experiment alginate is used as surfactant which isolates the spheres from air exposure.
- A microscope is used to control how the production is working and to adjust the speed of the injection machine.

MATERIALS:

EQUIPMENT

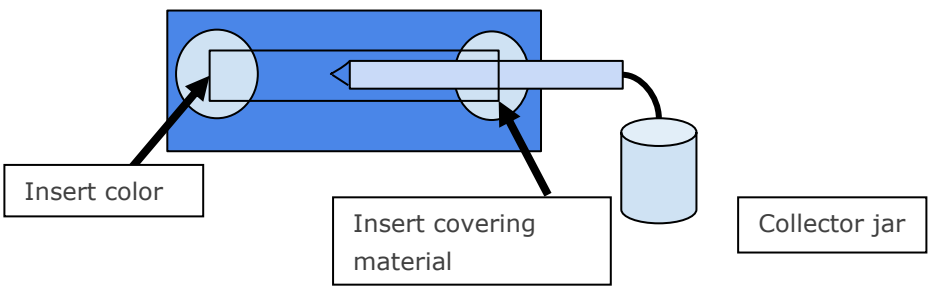
- Microfluidic chips done before
- Machine for injection of liquids by a controlled flow. (pumps at the speed wanted) (injection by syringe)
- 2 syringes to pour the liquid in and use it in the injection machine
- Tubes to connect the syringe tip of the machine tip to the syringe tip of the chip.
- Optic microscope to see the bubble production
- Spinnaker software and camera to see through the PC what we see through the microscope (not compulsory)
- A jar to collect the bubbles made

CONSUMABLES

- Inside the syringes:
  - One with your color/pigment
  - Another with your covering material
- Water to flow in whenever you want to clean up the chip.

PROTOCOL:

1. Turn on the microscope, the software, and introduce both liquids in the syringes
2. Take those syringes, introduce them in the injection machine and adjust it for a correct functioning.
3. Place the chip in the microscope making able the vision of the sharp-ended capillary tip
4. With the tubes, connect the colour syringe and the denser liquid as the picture shows.
5. Adjust the correct speed to the pumping machines in order to create uniform bubbles in a long period of time. Try with both tensioactives (Alginate and mineral oil) and see which works better on your pigment. The flow of spheres will exit through the square-ended capillary, so add a collector jar at the end of it.
6. In order to clean the chip to reuse, must add to both tubes connected to the chip syringes with sterilized water and pump it carefully and clean it as best as possible.



C. OTHER BUBBLE EXPERIMENTS

i. ALGINATE SPHERIFICATION

- In order to keep the colour away from O2, the creation of little spheres covered by an insulator as the alginate is a great option. But instead of making it through microfluidics, it can also be made by hand, as if making spherifications in a kitchen, it makes the same function but in a laboratory.

MATERIALS:

EQUIPMENT

- Eppendorf tubes
- falcon tubes
- Pasteur pipette
- magnetic stirrer
- beaker

CONSUMIBLES

- ink samples
- CaCl2
- Alginate
- H2O d

PROCEDURE :

1. Prepare the alginate by mixing 50 mL H2O d with 0,5g of alginate.
2. Mix the alginate in a magnetic stirrer.
3. Prepare the inks by mixing 1 mL of ink with 0,02 of CaCl2.
4. Place the alginate mixture on a beaker.
5. Take the ink sample with a Pasteur pipette and let drops fall onto the alginate 30s.
6. Place the alginate balls carefully on a storing tube with H2Od.

ii. PROTOCOL FOR EGG LIPID EXTRACTION

- Working with chemical surfactants stops the inks from being natural. This problem is solved by using the egg lipids as surfactant. The amphipathic molecules of the lipids are placed around the spheres making them more resistant.

MATERIALS:

EQUIPMENT

- bowl
- vortex
- centrifuge
- pipette and pipette tips
- falcon tube 50 mL

CONSUMIBLES

- egg yolk
- H2O d
- methanol
- chloroform

PROCEDURE :

1. Place the egg yolk on a 50 mL falcon.
2. Add 1 mL of H2Od.
3. Mix using the vortex.
4. Add 2 mL of methanol and mix gently.
5. Add 4 mL of chloroform and mix strongly.
6. Centrifuge 5
7. minutes at 2500 rpm.