

# Biosensitive tattoos for monitoring hormones concentration levels on real time: a new developing approach

Joan Campaña

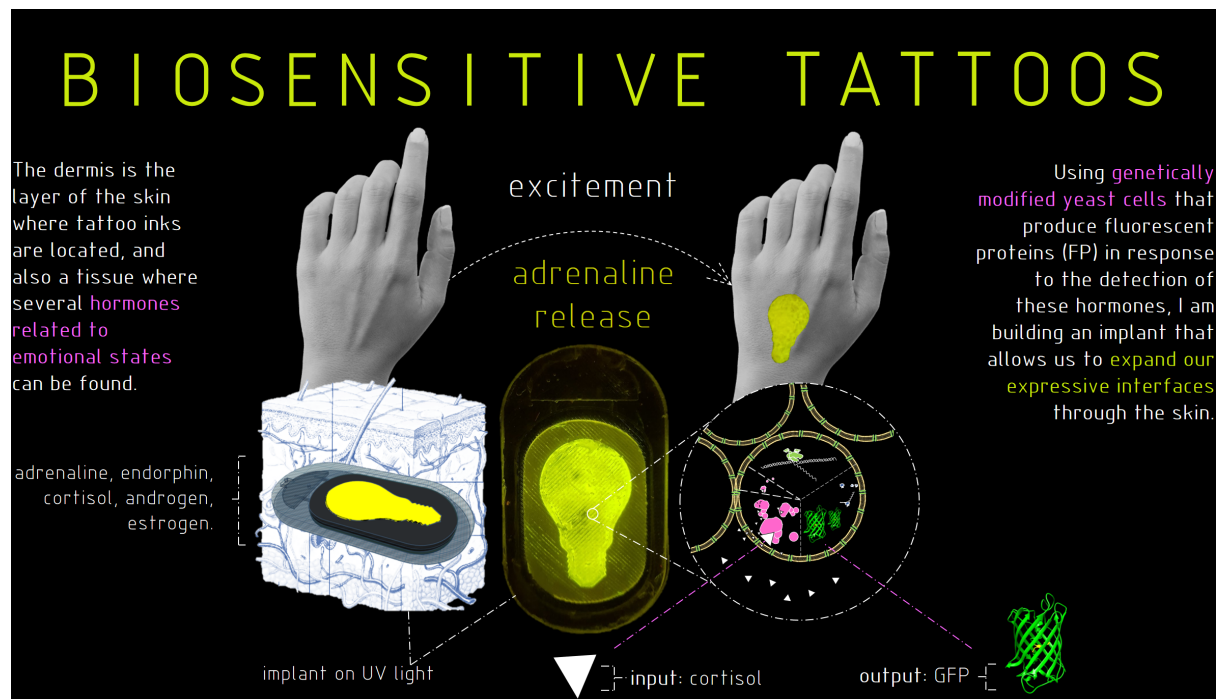
Bio Academy Lima  
Lima, Perú  
joana56cd@gmail.com

## Abstract

Chronic stress is one of major causes of important disorders and risk-conditions on health, such as heart diseases. Nonetheless, people who suffer it often overlook the stressful circumstances, until health damage is evident. Monitoring the levels of our hormones on real-time is a prospective solution. The presented prototype, a dermal implant, is the first step forward real-time hormones concentration measurement. It takes advantage of the endocrinology properties of the second layer of the skin, the dermis, where real tattoo inks are located. In this layer, several hormones (as cortisol, beta-endorphin, estrogens, androgens, etc.) related to emotional states can be measured. The implant is comprised by a scaffold made of the biocompatible materials PDMS and hydrogel, which contains genetically-modified yeast cells that in response to the detection of specific hormones concentration activates transcription of fluorescent proteins like GFP, YFP and RFP. The PDMS part of the scaffold includes a nutrients supply system for ensure cells nutritional requirements on the long term. Besides, the hydrogel layer has the crucial function of letting the hormones to diffuse through it to reach to the modified cells, while keeping the cells into the implant. This paper will compile the up-to-date development of the project.

## Keywords

Biotattoos, synthetic biology, dermal implant, hormones, fluorescent proteins



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## 1 The Dermis

When we get a tattoo, inks are positioned at the second layer of the skin, the dermis. This layer is very exciting from an endocrinologic point of view, due to the great amount of hormones closely related to emotional states that can be measured in its interstitial fluid. For example, endorphin is often associated with joyfulness and fun.

Compartment	Hormones and neurotransmitter repertoire
Epidermis	Vitamin D, PTHrP, androgens, T <sub>3</sub> , L-DOPA, catecholamines, acetylcholine, serotonin, glutamate, aspartate, CRH, urocortin, $\alpha$ -, $\beta$ -, $\gamma$ -MSH, ACTH, $\beta$ -endorphin, enkephalins, TRH
Dermis and adnexal structures	Vitamin D, PTHrP, <b>estrogens, androgens</b> , L-DOPA, serotonin, glutamate, aspartate, CRH, urocortin, $\alpha$ -, $\beta$ -, $\gamma$ -MSH, ACTH, <b><math>\beta</math>-endorphin</b> , enkephalins, GH, histamine catecholamines, acetylcholine <sup>a</sup>

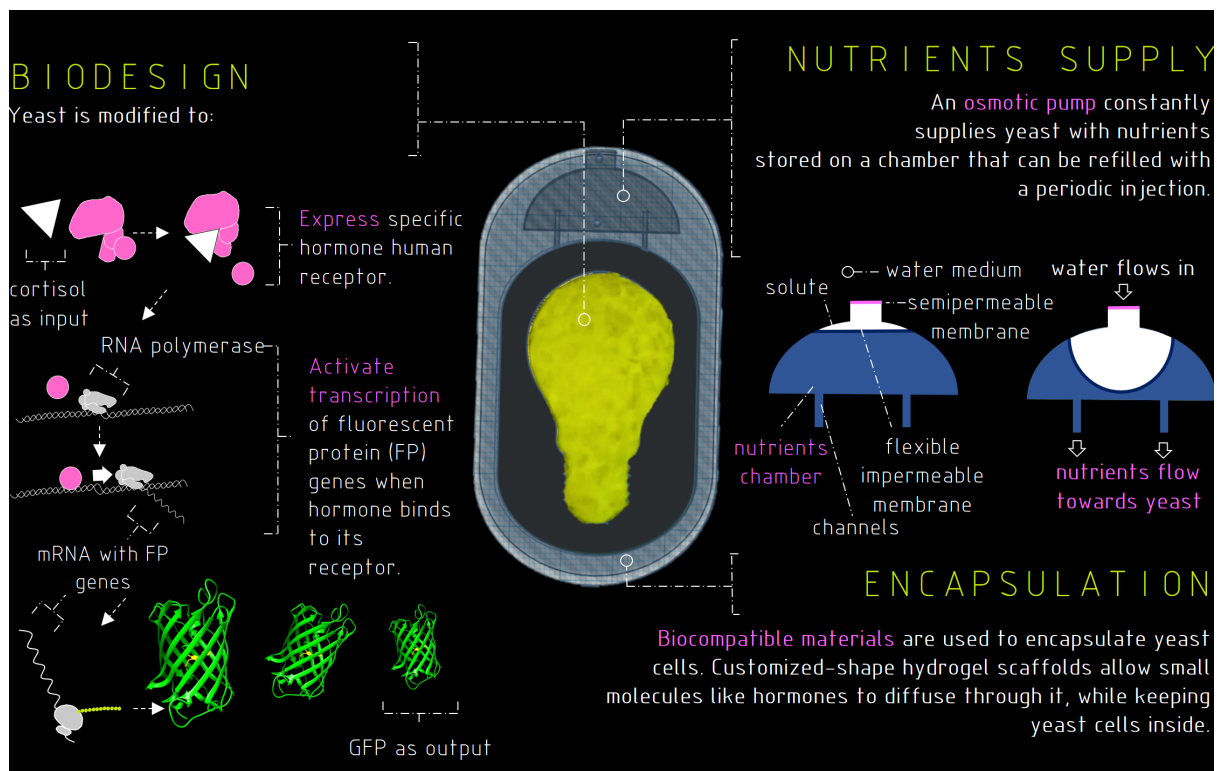
<sup>a</sup> In the dermis, catecholamines and acetylcholine originate predominantly from cutaneous nerve endings.

**Hormones at the dermis**

Neuroendocrinology of the Skin  
Andrzej Slominski – Jacobo Wortsman

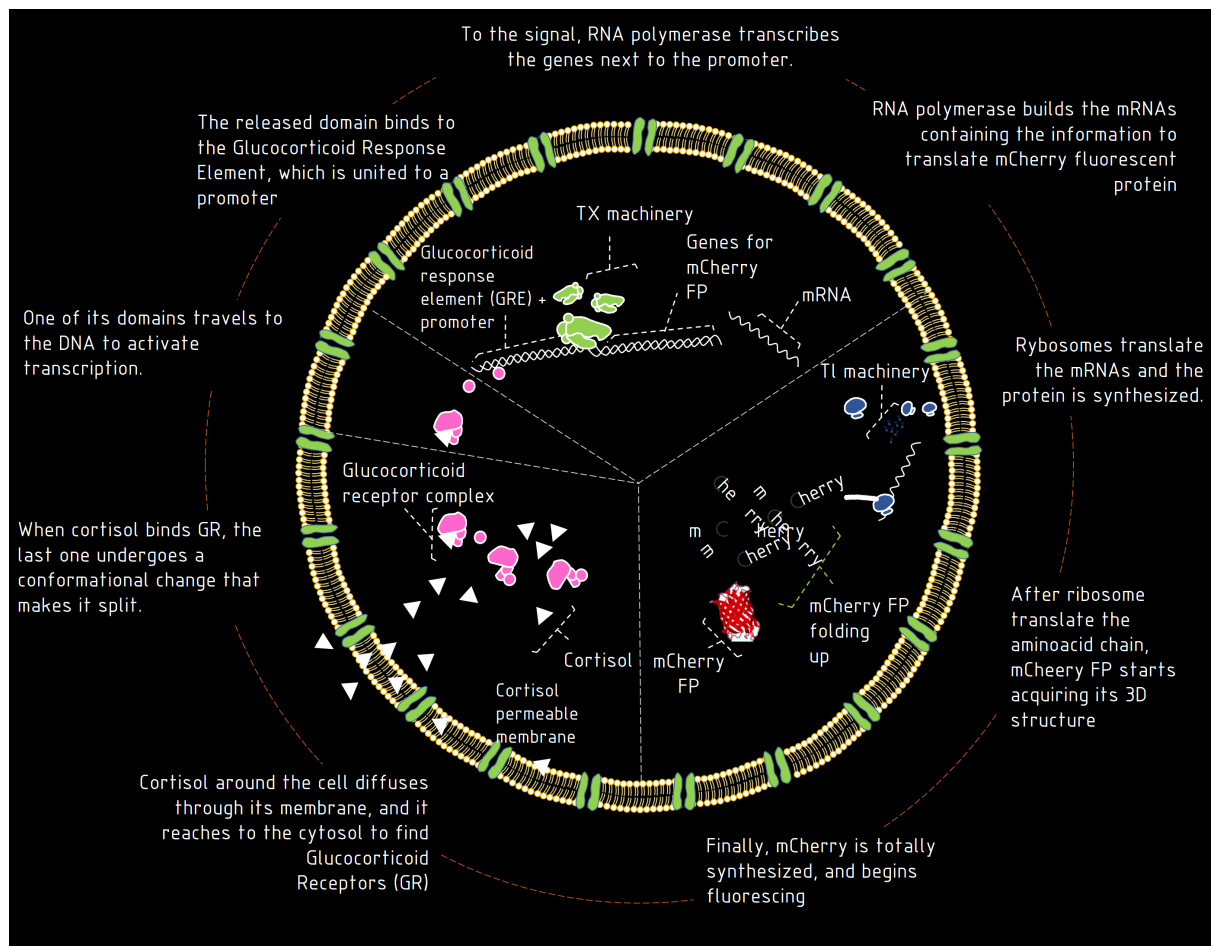
## 2 Overview of Biosensitive Tagoo Implant Parts

The biosensitive tattoo implant is divided on three major areas: Bio design, Nutrients supply and Encapsulation. I will share my up-to-date development on every area when I delve into each of them below.



Yeast cells were selected as hosts because they are eukaryotic organisms widely used on synthetic biology, that give me the possibility to work with complex molecular parts, like multidomain proteins. This is the design of a system for a modified yeast cell that recognizes cortisol as input and mCherry Red Fluorescent Protein as output:



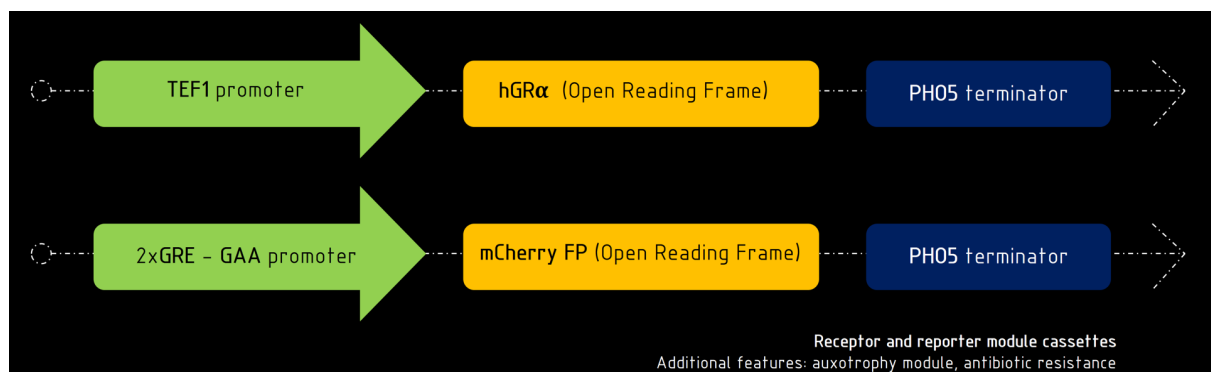


To build the system, yeast cells have to be modified in four modules:

- Receptor Module
- Reporter Module
- Auxotrophy Module
- Antibiotic Resistance Module

The first two modules are indispensable for the biosystem to function, while the other two are necessary to ensure the safety of the implant. Here the ones addressed are the receptor and the reporter module.

### 3 Bio Design



#### 4.1 Cassettes Elements

- **TEF1 promoter:** It is a promoter derived from *Saccharomyces cerevisiae*
- **hGRα gene (Open Reading Frame):** The NR3C1 gene codes for the human glucocorticoid receptor, and it is adapted to the usage codon of *Saccharomyces cerevisiae*

- **PHO5 terminator:** It is a terminator that comes from a common yeast strain: *Saccharomyces cerevisiae* (Arima et al. 1983).
- **GRE (Glucocorticoid response element):** It is the DNA sequence that detects the signal sent by the glucocorticoid receptors

Glucocorticoid transcription stimulation mechanism
Upon exposure to glucocorticoid, the receptor binds to the steroid, which results in the dissociation of hsp90, one of its domains. This leaves the receptor free to dimerize and to move to the nucleus, where the dimer binds to the GRE. This binding takes place via specific regions of the receptor known as the DNA-binding domain and the activation domain. The bound receptor then interacts with the basal transcriptional complex of RNA polymerase and associated factors to stimulate transcription of genes, by making closer the RNA polymerase with the promoter next to the GRE.

- **GAA promoter:** It is the glucoamylase promoter induced by maltose, from *Arxula adeninivorans*.
- **mCherry (Open Reading Frame):** This DNA sequence codes for the red fluorescent protein mCherry.

Single stranded mCherry gene
Translation start codon: AUG Translation stop codon: UAA Based on Part:BBa_J06504 - iGEM.
>BBa_J06504 part-only sequence (714 bp) ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACATGGAGGG CTCCGTGAACGGCCACGAGTTCGAGA TCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGG CCCCGTGCCCTTCGCTGGGACATCTGTGC CCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCACCCGCGGACATCCCCGACTACTTGAAGCTGTCC TTCCCCGAGGGCTTCAAGTGGGAGCGC GTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCTTGACAGGACGGCGAGTTCAT CTACAAGGTGAAGCTGCGCGGCACCAACT TCCCCCTCGACGGCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCC GAGGACGGCGCCCTGAAGGGCGAGATCAA GCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGACCCTACAAGGCCAAGAAG CCCGTGACGCTGCGCGGCCTACAACGTC AACATCAAGTTGGACATCACCTCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGC CGCCACTCCACCGCGGCATGGACGAGC TGTACAAGTAATAA

- **Auxotrophy module:** Controlling where are modified cells able to grow by forcing them to have a nutritive requirement that they cannot synthesize.
- **Antibiotic resistance module:** Avoiding other species growing in the medium by putting an antibiotic in the medium that only the mutants are resistant to.

### 3.1 Plasmid Construction

#### 3.1.1 Receptor Module

Steps overview:

- Extracting hGR $\alpha$  gene
- Inserting gene in backbone plasmid pBS-TEF1 promoter-PHO5 terminator
- Inserting cassette in a carrier plasmid

To insert the hGR $\alpha$  gene into *Saccharomyces cerevisiae* is required flanking the ORF directly with EcoRI and BamHI cleavage sites by in vitro mutagenesis. These oligonucleotides are used as primers and the pCMV-GR11 containing the hGR $\alpha$  gene serves as the template in the PCR. The EcoRI–BamHI-flanked hER $\alpha$  gene fragment is inserted into the plasmid pBS-TEF-PHO5 between the strong constitutive *Arxula adeninivorans*-derived TEF1 promoter and the *Saccharomyces cerevisiae*-derived PHO5 terminator. The expression cassette TEF1 promoter-hGR $\alpha$  gene-PHO5 terminator constructed is then inserted into the basic pAL-HPH1 plasmid and the resulting construct, pAL-HPH-hER, used to transform *Saccharomyces cerevisiae*.

Primers: Addgene Sequencing Result

Plasmids: pCMV-GR11, pBS-TEF-PHO5, pAL-HPH1

Restriction enzymes: EcoRI and BamHI

Equipment: Thermocycler

### 3.1.2 Reporter Module

Steps overview:

- Inserting mCherry gene in backbone plasmid pBS-TEF1 promoter-PHO5 terminator
- Building 2xGRE-GAA promoter
- Replacing TEF1 by 2xGRE-GAA on the first plasmid

#### 1. Extraction and insertion of mCherry gene on backbone plasmid

To insert the mCherry gene into *Arxula adeninivorans* is required flanking the ORF directly with EcoRI and BamHI cleavage sites by in vitro mutagenesis. The oligonucleotides 5'- GAATTCatggcgtggctggtc -3 (EcoRI restriction site in capitals) and 5'- GGATCCttaggcggacactatggcta -3 (BamHI restriction site in capitals) are used as primers and pTH760-CEN-mCherry\_v3 plasmid containing the mCherry gene serves as the template in the PCR. The EcoRI–BamHI-flanked mCherry gene fragment is inserted into the plasmid pBS-TEF-PHO5 (probably between the strong constitutive *Arxula adeninivorans*-derived Sall–EcoRI flanked TEF1 promoter and the *Saccharomyces cerevisiae*-derived PHO5 terminator).

Primers: 5 -GAATTCATGACCATGACCCTCCAC-3, 5 -GGATCCGCCAGGGAGCTCTCAGA-3

Plasmid: pTH760-CEN-mCherry\_v3, pBS-TEF-PHO5

Restriction enzymes: EcoRI and BamHI

Equipment: Thermocycler

#### 2. Promoter construction

Now we build the GAA promoter glucoamylase promoter induced by maltose, containing two copies of Glucocorticoid Response Element. GREs with the nucleotide sequence 5'-GGTACAGGATGTTCT-3 are selectively retrieved from chicken genome (using the strategy of Klinge, 2001). They are also integrated into positions –203 of the GAA promoter by PCR and sliced by overlap extension (SOE) mutagenesis using the primers MFERE203a (5 -GGTACAGGATGTTCTGGTACAGGATGTTCTTGAGATAA-3 from –203 to –165) and MFERE203b (5 -GGTACAGGATGTTCTGGTACAGGATGTTCTTTTATCC-3 from –233 to –196). The resulting DNA fragments are digested with Sall–EcoRI and gel-purified.

Primers: MFERE203a (5 -GGTACAGGATGTTCTGGTACAGGATGTTCTTGAGATAA-3) and MFERE203b (5 -GGTACAGGATGTTCTGGTACAGGATGTTCTTTTATCC-3)

DNA sequence: GREs with the nucleotide sequence 5'-GGTACAGGATGTTCT-3 from chicken genome.

Restriction enzymes: Sall–EcoRI

Equipment: Thermocycler

### 3. Replacing TEF1 by 2xGRE-GAA

The vector is constructed by replacing the Sall–EcoRI flanked TEF1 promoter of the plasmid pBS-TEF-mCherry-PHO5 (built on phase I) with the Sall–EcoRI-flanked *Arxula adenivorans*-derived GAA promoter (built on phase II) to form pBS-GAA-mCherry- PHO

DNA sequence: pBS-TEF-mCherry-PHO5 (built on phase I) and Sall–EcoRI-flanked *Arxula adenivorans*-derived GAA promoter (built on phase II)

Restriction enzymes: Sall–EcoRI

Equipment: Thermocycler

With this process, plasmids are complete.

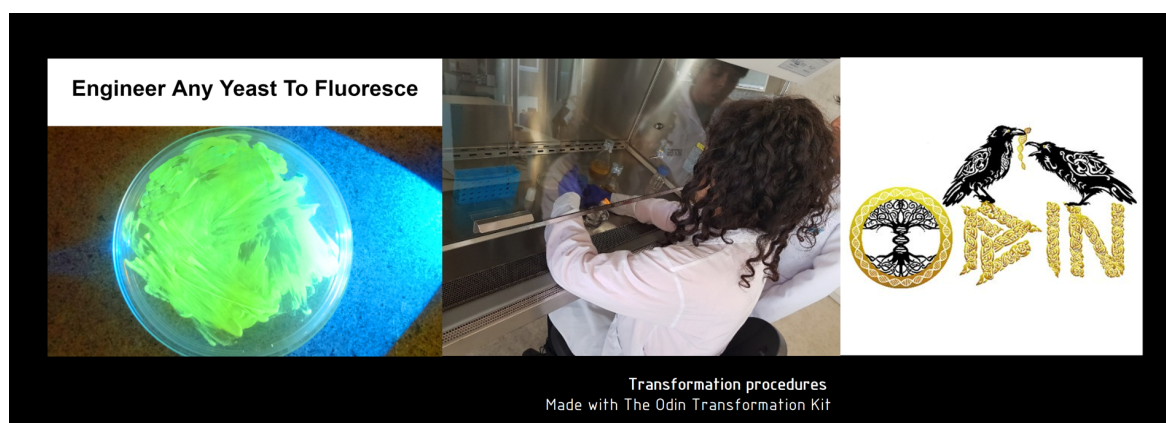
### 3.2 Gene Expression

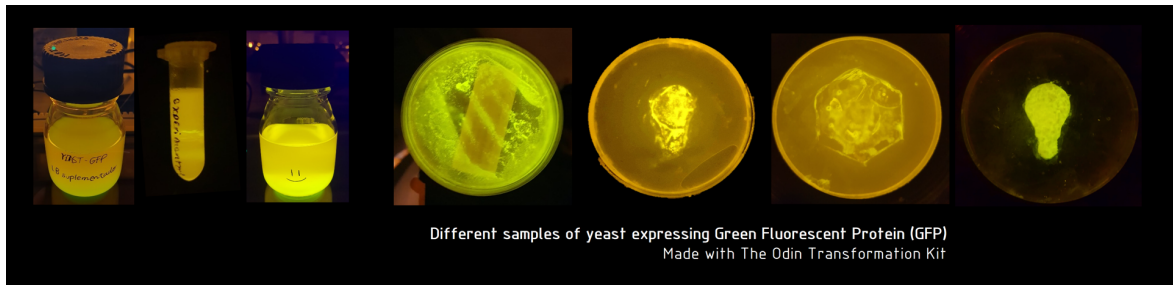
To express the genes, it is required to transform *Saccharomyces cerevisiae*, so the yeast transformation protocol of The Odin is equally useful. The following table shows the protocol steps and their corresponding description:

Mixing	Mix together sample of recently grown <i>Saccharomyces cerevisiae</i> , plasmid, and transformation mix on an Eppendorf tube.
Heat shock	Incubate the sample at 42°C water for one hour.
Adding media	Adding media Add YPD media to cell solution.
Incubation on tube	Incubate the sample on the tube at 30 °C water for four hour.
Plating	Plate 400uL of the yeast solution and let dry for 10 minutes
Incubation on dishes	Incubate the plate at 30 °C for 24-48 hours or room temperature for two days or more.
Observing and measuring results	Use a spectrophotometer to measure mCherry FP expression quantitatively.

### 3.3 Up-to-Date Development

To develop the current implants, yeast was transformed to express Green Fluorescent Protein using the transformation kit of The Odin. In relationship with my project, yeast in this kit has GFP as a reporter, features g418 antibiotic resistance.

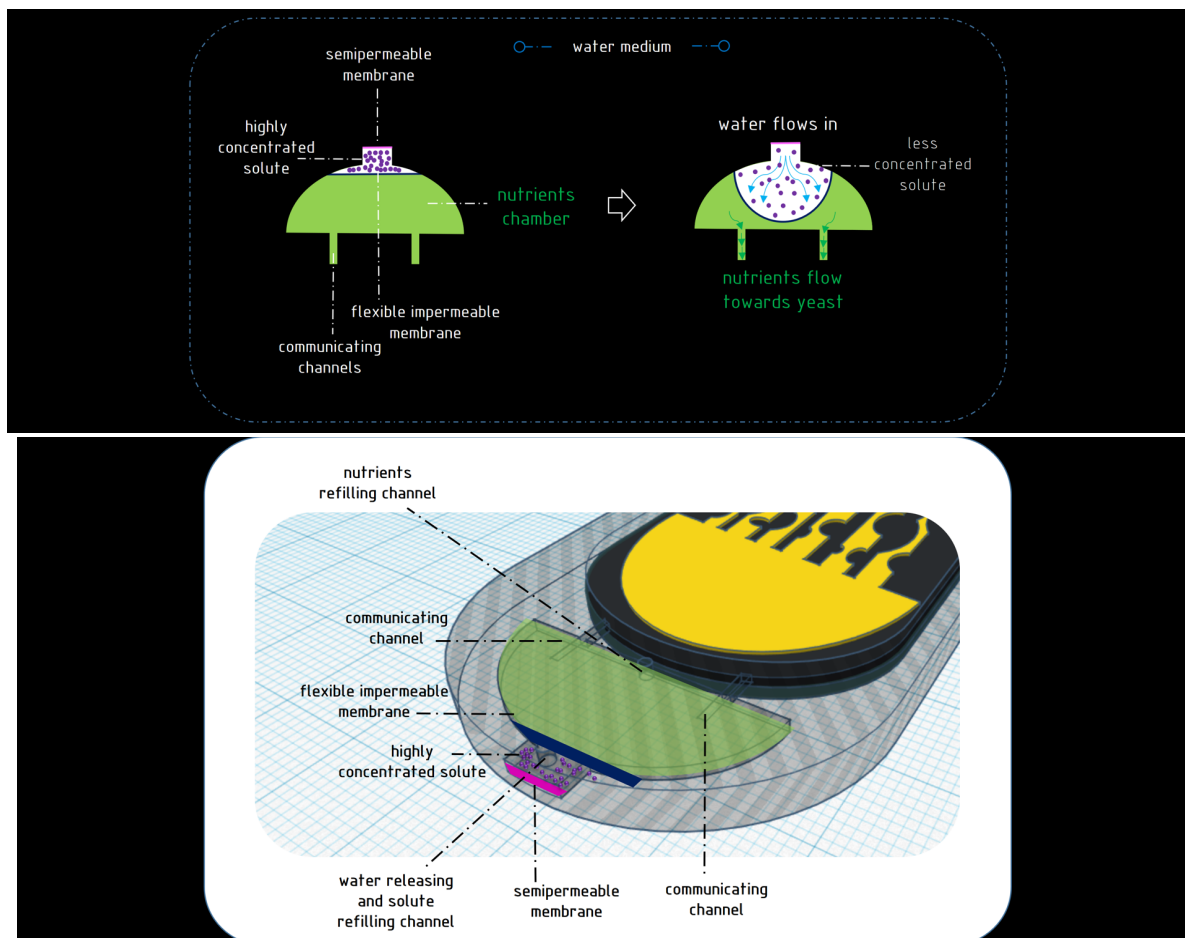




## 4 Nutrients Supply

### 4.1 Design

To take yeast cells into the dermis we must ensure they will be provided with all their nutrients requirements, as well as proper environmental conditions and social security. Yeast cells need O<sub>2</sub>, water, sugar and proteins, and they produce CO<sub>2</sub> as residue. The oxygen requirement and the carbon dioxide release are not a problem, since at the dermis and epidermis exists a phenomenon called cutaneous uptake of atmospheric oxygen on one side, and constant gas exchange on the other. Nonetheless, we must provide our yeast with water, sugar and proteins, so I designed the Yeast Osmotic Feeder, the microfluidic device that feeds the cells inside at a constant rate powered with a osmotic pump. This is how it works:



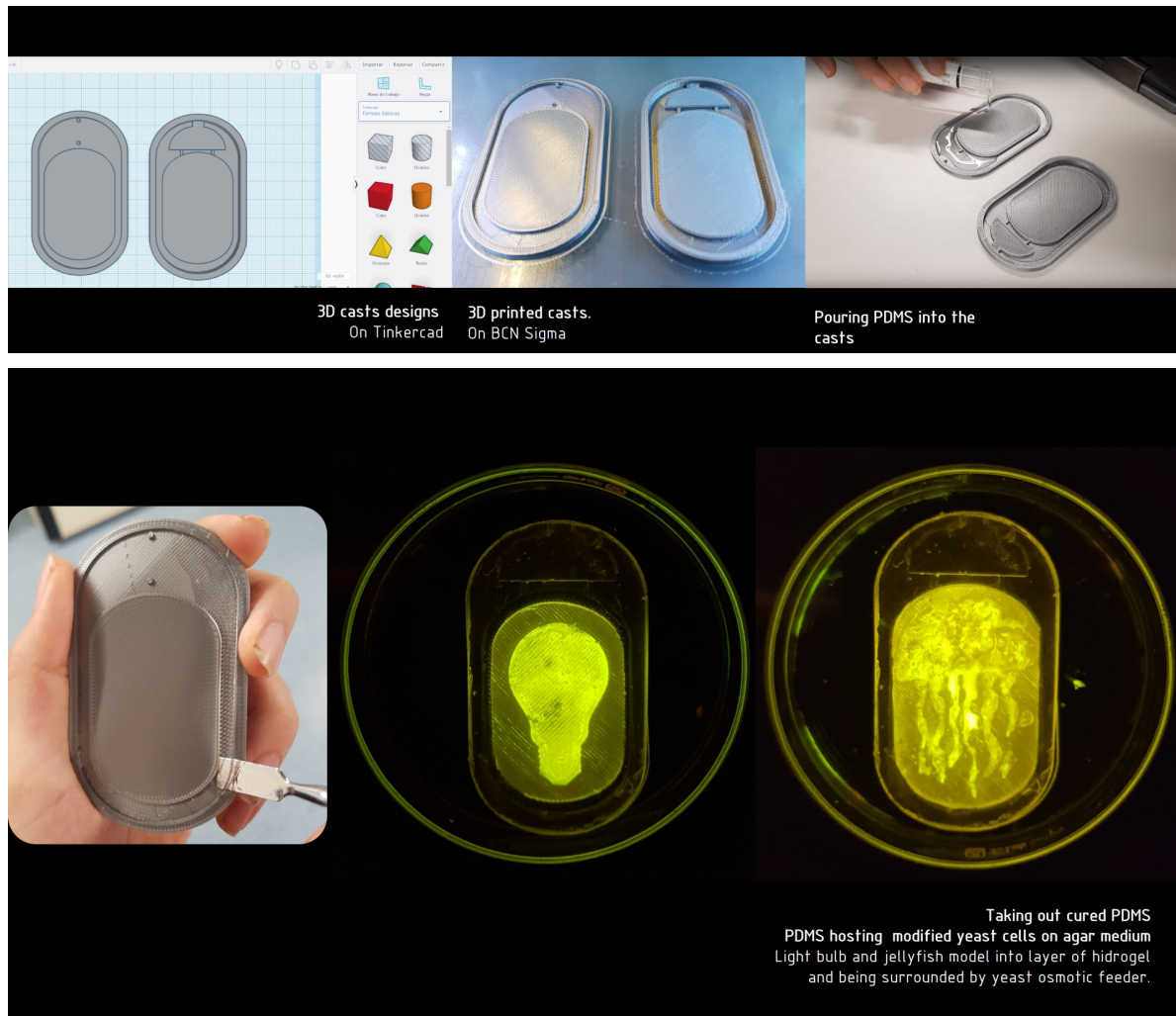
In the phenomenon of osmosis, water tends to move through a semipermeable membrane in order to equilibrate the solute concentrations in all the medium. In this case, as solute is highly concentrated on the first chamber, water tends to flow into it. Inside it will find a flexible non-permeable membrane, but it



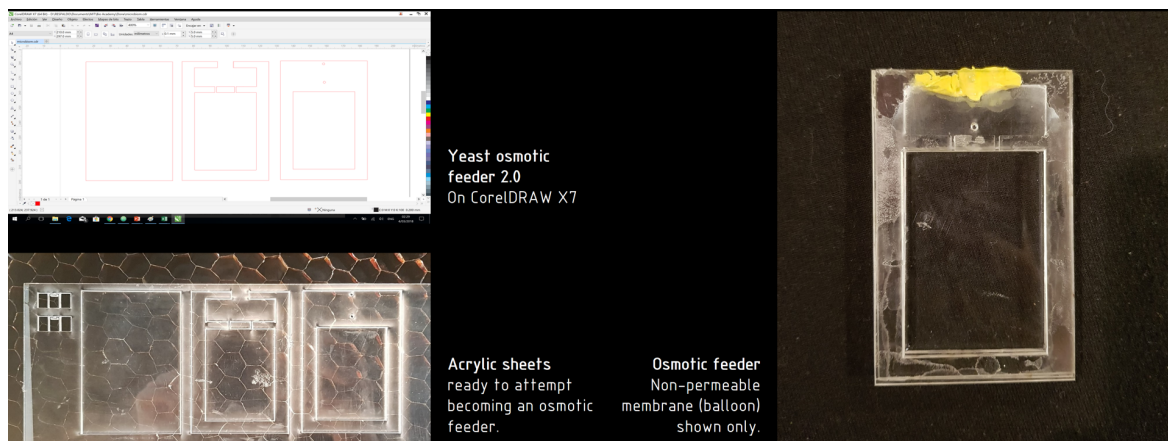
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will keep flowing in. This will push the flexible membrane inside the system, and therefore nutrients will be pushed too. Using the communicating channels, nutrients will move from the second chamber towards the cells.

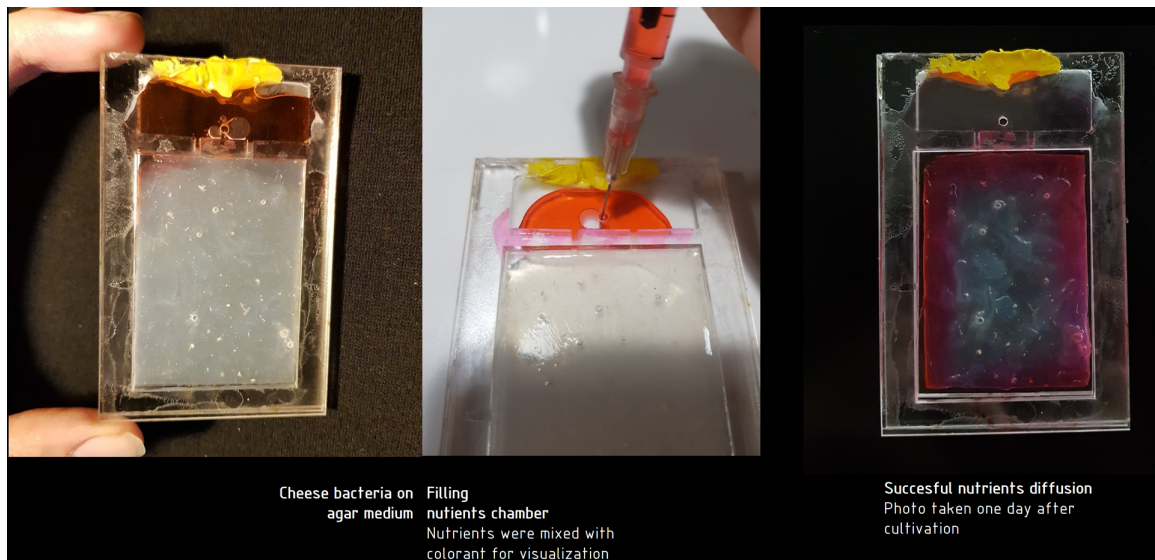
## 4.2 Up-to-Date Development



A test of the osmotic pump building the device with laser cutting in acrylic 2mm thick was carried out. I used the semipermeable membrane of a syringe filter, a piece of balloon as the flexible non-permeable membrane and salt as the solute. I had not considered the strong resistance balloons offer to be deformed, hence I am now looking for a non-permeable membrane that can indeed be moved by the power of water in osmotic pressure.







## 4.2 Instructions of Assembly

### 4.2.1 PDMS Casting

1. Either 3D-printed casts or acrylic casts can be used to mold PDMS. 3D model of the casts used are available on the Metafluidics page of the project, as Yeast Osmotic Feeder (<https://metafluidics.org/devices/yeast-osmotic-feeder/>). In case you decide for 3D-printing, print the model with full density.
2. On a beaker, mix PDMS (Sylgard 184) and its curing agent (10:1). The volume needed depends on what size you decided to make the device. It should be 12 mL of total volume if the size on the uploaded model remains unaltered (10 mL of PDMS and 2 mL of curing agent).
3. Pour the fresh PDMS into the casts and leave it at a stable surface for ~24 hours at room temperature or ~1.5 hours at 80 °C.
4. Retire gently the molded PDMS of the casts. Remember that the device has delicate parts that should be handled carefully.

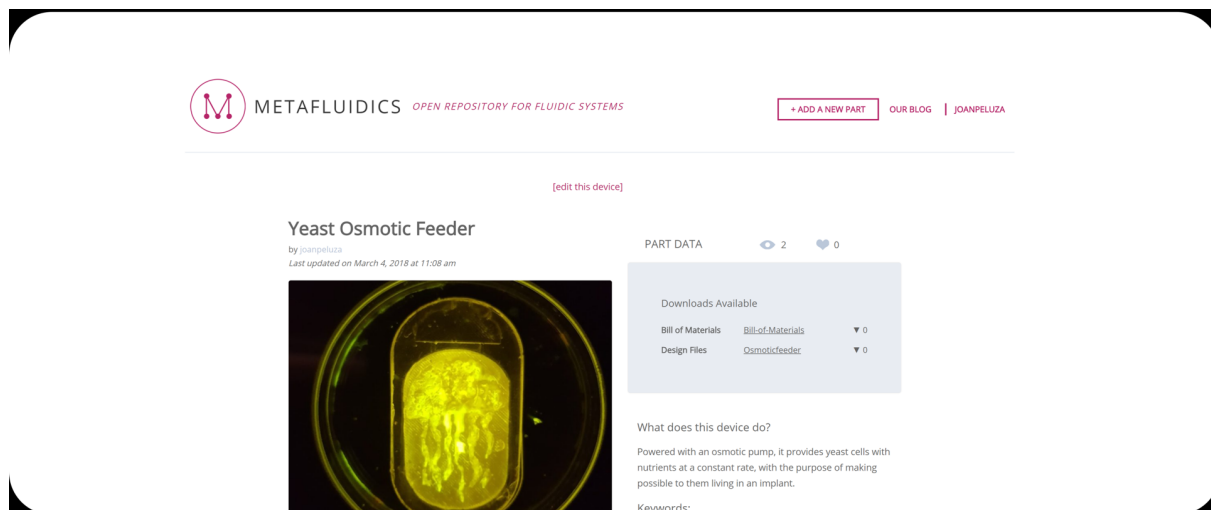
### 4.2.2 Membranes Positioning

1. Cut little a rectangle of Visking tube membrane to make the semipermeable membrane required. Cut a little rectangle of an elastic polymer (thin balloons are an option) to make the flexible impermeable membrane required.
2. Use super glue to firmly attach to their place both membranes to the first part of PDMS, as you can see in the schematic.

### 4.2.3 PDMS Adhesion

1. Apply adhesive tape to the surfaces of each PDMS part where they will touch one to the other.
2. Put superglue on the superior edge of the membranes, in order to have them attached to the the second part of PDMS when left ready to adhere.
3. Rapidly put the second part of PDMS and hold it until super glue is ready.
4. Bake the whole device at 65°C for 2 hours.
5. Retire it from the oven carefully, some parts might still be hot.

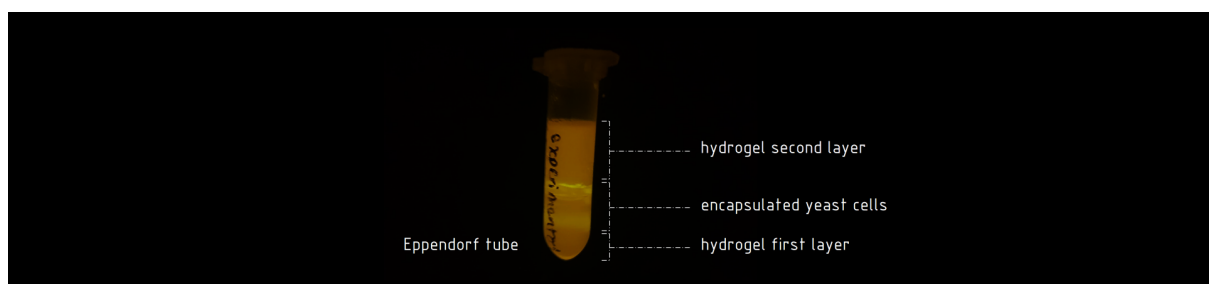
More information is available at Metafluidics as Yeast Osmotic Feeder.



## 5 Encapsulation

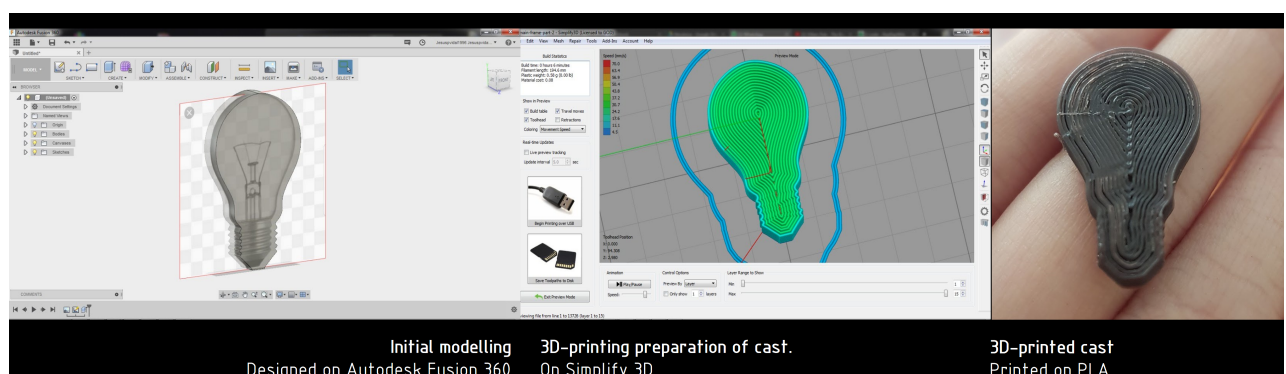
### 5.1 Preliminary Tests

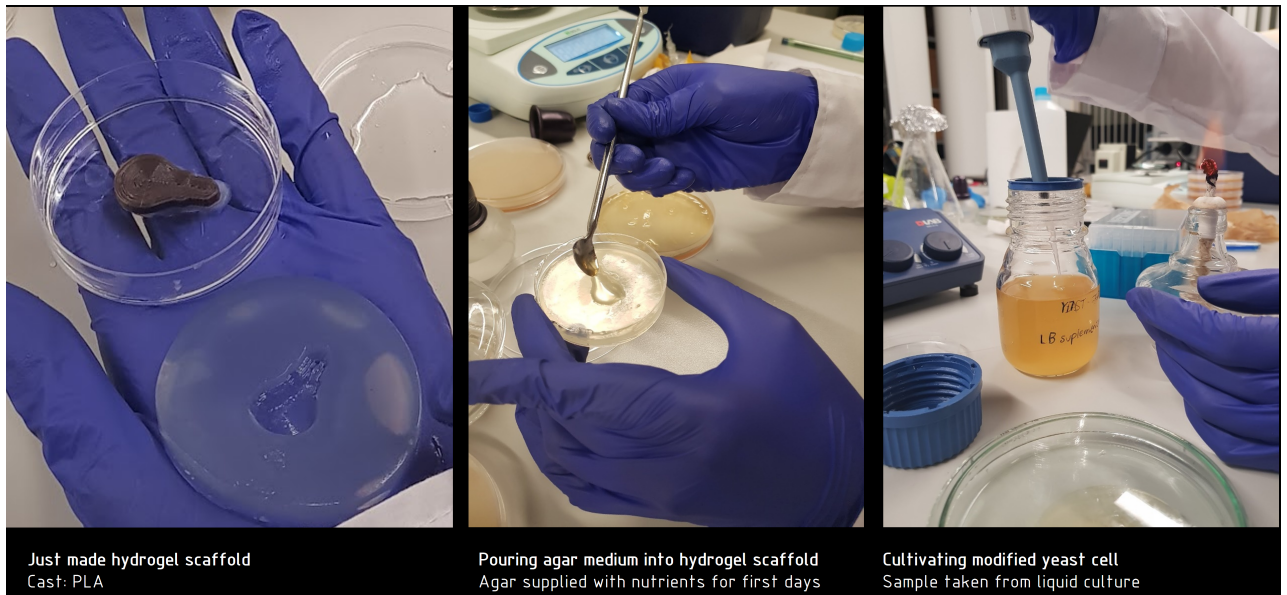
Hydrogel is a biocompatible material that allows small molecules to diffuse through it. This way, it allows molecules like cortisol or adrenaline to get to yeast cells, but prevents cells from going out and fight to the death with our immune system and FDA. This property was tested by putting cultivated fluorescent yeast medium between two layers of hydrogel in this Eppendorf tube.



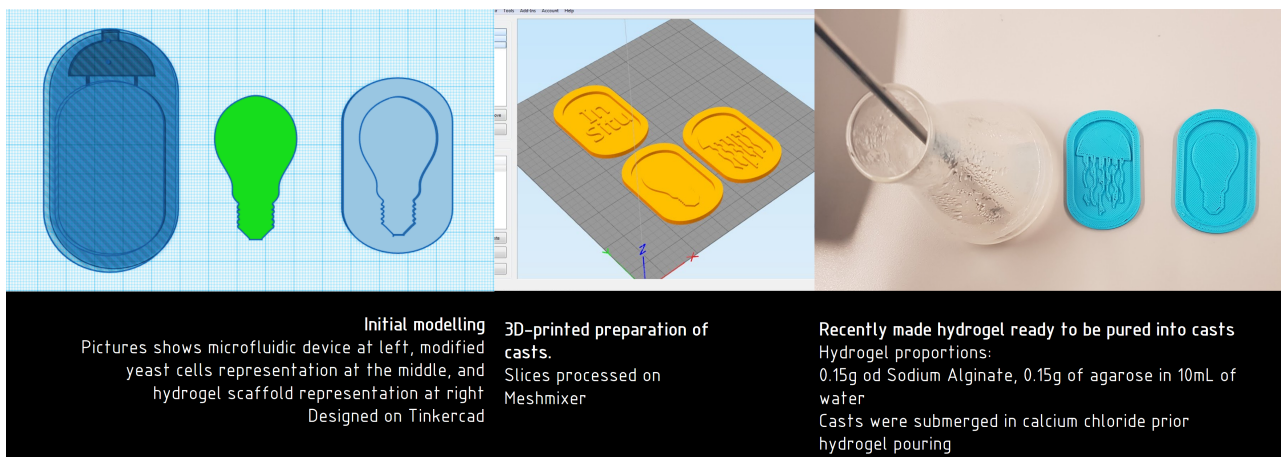
As you see, yeast keeps on its medium rather than diffusing through hydrogel. Besides, PDMS is also a biocompatible material, but it just allows the diffusion of gases like O<sub>2</sub> and CO<sub>2</sub>. Far from being a problem, this is our way to choose the shape of the fluorescent tattoos. PLA 3D printing and laser cutting are both very useful tools that allow us to achieve high-precision scaffolds.

### 4.3 Hydrogel Scaffold I





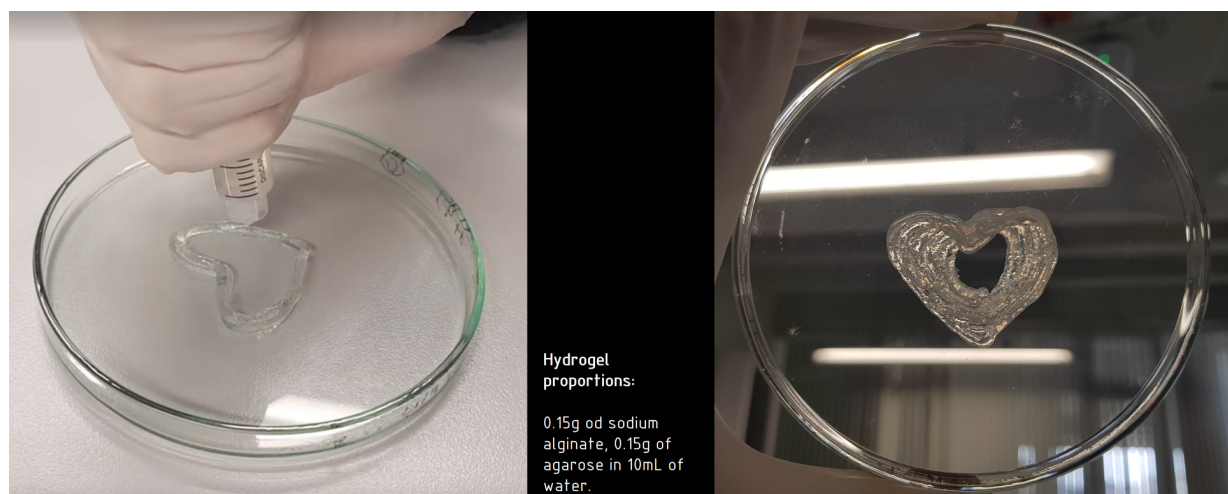
#### 4.4 Hydrogel Scaffold II



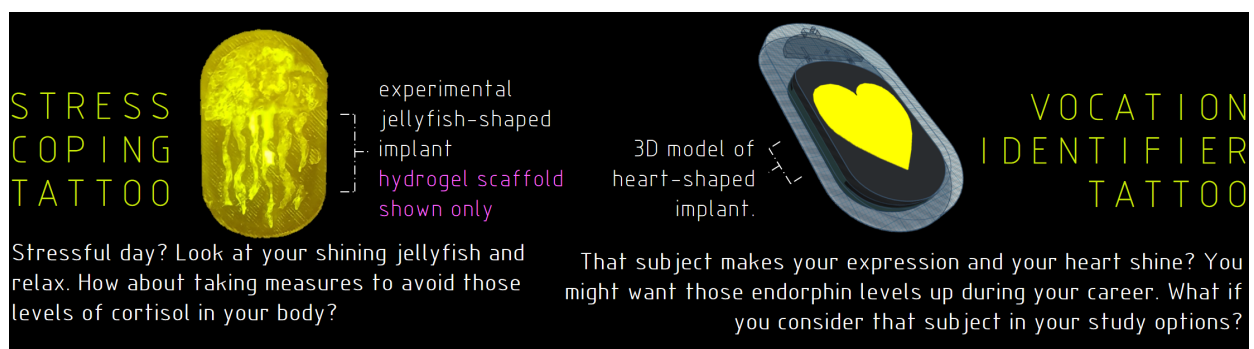




Neither agarose nor alginate are consumed by these cells, and since hydrogel is not supplemented with the nutritional requirement of the auxotrophic yeast, cells won't be able to grow there. Hydrogel is made principally from sodium alginate, a substance that solidify in contact with calcium chloride. Because of this property, it is often used on bioinks. The hydrogel that I prepared is made of sodium alginate and agarose (used proportions: 0.15g sodium alginate, 0.15g agarose in 10 mL of water), and its gel-like nature prior to cool until room temperature its ideal for using it as a bioink. In fact, it is part of many commercial bioinks. Here is the test made using the principle of 3d-printing with a syringe.



## 5 Applications



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## References

- A novel estrogen sensor based on recombinant *Arxula adenivorans* cells. Hahn T1, Tag K, Riedel K, Uhlig S, Baronian K, Gellissen G, Kunze G.
- Integrative transformation of the dimorphic yeast *Arxula adenivorans* LS3 based on hygromycin B resistance. Rösel H1, Kunze G.
- Simultaneous detection of three sex steroid hormone classes using a novel yeast-based biosensor. Chamas A1, Pham HTM1,2, Jähne M3, Hettwer K3, Uhlig S3, Simon K4, Einspanier A5, Baronian K6, Kunze G1.
- Engineer any yeast to fluoresce protocol