

A perfusable 3D printed device for scalable cultured meat production

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“Here's to the crazy ones. The misfits. The rebels. The troublemakers. The round pegs in the square holes. The ones who see things differently. They're not fond of rules. And they have no respect for the status quo. You can quote them, disagree with them, glorify or vilify them. About the only thing you can't do is ignore them. Because they change things. They push the human race forward. And while some may see them as the crazy ones, we see genius. Because the people who are crazy enough to think they can change the world, are the ones who do.”

Rob Siltanen

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This whole project was an audacious and stubborn decision of mine, stepping into an unknown field without any special support from an expert in it. However, if I managed to achieve its goals was only because a significant number of people have opened their arms and hearts to welcome me and help me in everything I needed.

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Abstract

Throughout history, humanity has been using science and technology to adapt to the most various challenges. Currently, one of the biggest challenges humanity faces is sustainability. The food sector, in particular, is abusing animals and the environment for feeding the world's growing population. According to FAO, by 2050, there will be almost 10 billion people on Earth and meat demand, in particular, will increase by 73%. This will soon become unsustainable and alternative ways of producing meat and animal protein-based products are urgently needed. Cellular agriculture, based on tissue-engineering principles, is one of the most promising technologies for "clean meat" production, without environmental pressures, animal welfare issues and public health concerns. However, cellular agriculture and cultured meat production are still in a very early stage, facing numerous challenges.

In this work, we aimed at tackling some of those challenges. In particular, a scalable solution for creating thick bioengineered tissue constructs was developed, based on the use of a perfusable device for dynamic 3D cell culture in edible scaffolds. Some attempts for establishing primary cultures of bovine muscle cells were also made. In order to achieve perfusion through thick (>5 mm) hydrogel-based scaffolds, we have designed a perfusable scaffold holder that was fabricated by 3D printing. After 12 iterations, the optimized final version consisted of a cylindric shaped device with porous canals, that was attached to the spindle of a spinner flask for magnetic stirring, inducing mass transport by convection. The device successfully allowed dynamic 3D culture of C2C12 myoblasts within a 6 mm-thick alginate disc, with cells remaining alive for at least 3 days, across the whole disc thickness. Although not ideal, alginate hydrogels were used as an animal-free and food-grade scaffolding material, to validate the perfusion device and hold cells in place, as in a tissue. Regarding cell sourcing, validation tests have been performed with a mouse myoblast C2C12 cell line, which would be unsuitable for producing human edible meat. Therefore, we have also tried to isolate and expand two populations of bovine muscle cells: whole-population muscle cells and sorted satellite cells. The latter apparently showed higher differentiation potential for muscle fibers formation, upon induction. Collectively, these results represent a step forward towards solving the challenge of maintaining cells alive in thick engineered-tissues, which is key for the production of cultured meat products as a sustainable alternative to conventional meat.

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Abbreviation List

2D	Two dimensional
3D	Three dimensional
BSA	Bovine Serum Albumin
CDC	Center for Disease Control and Prevention
CM	Conditioned Medium/Media
DbC	Distance between channels
DMEM	Dulbecco's Modified Eagle Medium
Ecd	External channel diameter
ECM	Extracellular Matrix
ESC	Embryonic Stem Cells
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FSC	Forward Scatter
GHG	Greenhouse Gases
GWP	Global Warming Potential
HS	Horse Serum
LCA	Life-cycle assessment
Icd	Internal channel diameter
IP	Intellectual Property
iPSC	induced Pluripotent Stem Cells
PBS	Phosphate-Buffered Saline
P/S	penicillin/streptomycin
RBC	Red Blood Cells
RFU	Relative Fluorescence Units
RGD	(Glycine)4-Arginine-Glycine-Aspartic acid-Serine-Proline
SC	Satellite Cells
SSC	Side Scatter
TE	Tissue Engineering
USA/US	United States of America/United States
USDA	United States Department of Agriculture
US PIRG Education Fund	US Public Interest Research Group Education Fund

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Introduction

1. Science and technology as natural ways of evolution

To contextualize this work, it is important to start by looking back at history and recalling how humans have used science and technology to reduce their dependence on animals throughout time. Humanity used to depend on animals for almost everything: clothing, tools, fuel, shelter and transportation. Fortunately, new and better ways to dramatically replace animals in all these areas have been found. However, there is still one major area where very little has changed: Food.

In the nineteenth century, societies around the world relied massively on whale oil both as a fuel for lightning, heating and cooking, as well as a mechanical lubricant source. According to the New York Times¹, the whale oil industry was, at that time, the fifth largest industry in the United States of America (USA), with more than 700 ships responsible for slaughtering more than 8000 whales in 1853. However, the whaling industry was rapidly decimated, down to only 200 ships in 1890 and zero in 1971². Among other factors, the invention of kerosene was one major factor for this decline, as it offered a much better and affordable alternative to whale oil³. It was also eventually replaced by Thomas Edison's electric light bulb, as human societies started using electricity.

Whereas whale oil may not sound relatable enough to many people nowadays, horses are certainly a far more familiar example of animal exploitation for human use. In modern societies, and in many places around the world, horses are still used for transportation, not only by farmer workers, but also by local authorities and for touristic purposes. However, in the past, horses played a much prominent role in food, transportation, communication, agriculture and commerce, all over the world⁴. Yet, increasing population growth rates made that rapidly progress towards unsustainability. In fact, as described by Jeff Stibel⁵, the US government predicted, back in 1880, that New York City would cease to exist within 100 years due to the insane amounts of manure produced⁶ by the six million horses needed to cope with the increasing demand for equine labor within that timeframe. In the end, what eventually dictated horses' replacement were not environmental reasons, nor animal empathy, but the invention of internal combustion engines. Back then, people didn't know about cars and trucks

and never imagined them to replace horses, although they needed them.

The same way kerosene (and electricity later) and internal combustion engines played an important role in replacing animals in the fuel and transportations sectors, should we expect alternatives to animal-derived food? Can animals be replaced in the only area they are still being massively used today?

There is no doubt that, as long as people continue to demand for meat and other animal products, the same way they kept and keep demanding for fuel and transportation, the market has to ensure the supply. The problem relies on the fact that, currently, animal farming is the only way humans have to accomplish that. Yet, just like the horses, this will eventually become unsustainable, as the global population keeps growing exponentially.

2. Meat consumption and the impact of animal farming

According to the Food and Agriculture Organization of the United Nations (FAO), world population is expected to grow from 7.7 billion people today to 9.6 billion in 2050⁷. Along with rising incomes, urbanization and enrichment of developing countries economies', diets around the world are expected to become even richer in animal products such as meat. In fact, FAO estimates that demand for meat and milk will grow 73% and 58%, respectively, by 2050 in comparison to 2010⁷, while natural resources such as water, land and energy will not cope with this demand. It is not surprising that the exhaustion of these resources is a natural consequence of an overgrowing world population, yet there is an urgent need to adapt and evolve to more sustainable ways of dealing with these issues.

As early as 2006, the impact of animal farming on the environment and human health was already very well known. FAO's first report⁸ on this matter thoroughly described the pernicious consequences of massive farming in land degradation, water, atmosphere and climate, as well as in biodiversity. Currently, the livestock sector is by far the largest anthropogenic user of land, occupying 30% of the entire planet's ice-free terrestrial land, and being responsible for the transformation of 70% of Amazon's forest land into pastures⁸, which places it as one of the key accountable factors for our planet's deforestation.

Climate change is, without a doubt, a real concern nowadays with the largest countries in the world signing accords, such as the famous Paris Agreement⁹, to keep global warming

below 2 °C. Surprisingly, little attention is given to the impact of industrial animal farming impact on climate, even though livestock contributes with more greenhouse gas (GHG) emissions (18% of all emissions) than the entire transportation sector⁸. Regarding anthropogenic emissions, animal farming is responsible for 37% of methane (with 23 times the global warming potential (GWP) of CO₂), 65% of nitrous oxide (with 296 times the GWP of CO₂) and 64% of ammonia emissions, which contribute to acid rain and ecosystems' acidification. In fact, if global trends on meat and dairy consumption don't decrease, global mean temperatures will likely rise more than 2 °C, even with drastic reductions across other non-agricultural sectors¹⁰. Again, none of this matter is contemplated in any of the international agreements on climate change.

On the other hand, water is essential for life and, while Earth is 70% water, humanity only gets to use around 2%¹¹. If a few decades ago, no one would even consider the idea of running out of fresh water, that shocking reality almost took Cape Town in South Africa without a warning¹². FAO estimates that 64% of the world's population will live in water-stress conditions by 2025⁸. Again, livestock has a major impact, as it uses 8% of global human-usable water and is also considered to be the largest sectoral source of water pollution, "contributing to eutrophication, dead zones in coastal areas, degradation of coral reefs, human health problems and emergence of antibiotic resistance", as reported by FAO⁸. Looking at the numbers, there is no denial about how serious the issue is: Table 1 shows the total water footprint for several vegetable and animal origin products, as estimated by Mekonnen and Hoekstra¹³. For instance, to produce one kilogram of beef, around 15.000L of water are spent, whereas the same amount of water can produce almost 4kg of pulses (e.g. beans, lentils), which contain 1.5 times more protein than beef¹³. Actually, pulses are the food product with the most optimal water usage per gram of protein, requiring 19L to produce 1 gram of protein.

Table 1 - The water footprint for vegetable and animal food products adapted from Mekonnen and Hoekstra¹³.

Food product	Water footprint (L/kg)	Protein content (g/kg)	Water footprint/g of protein (L/g of protein)
Vegetables	197	12	26
Cereals	1644	80	21
Pulses	4055	215	19
Nuts	9063	65	139

Milk	1020	33	31
Eggs	3265	111	29
Chicken	4325	127	34
Pork	5988	105	57
Beef	15415	138	112

Regarding biodiversity, if livestock is a major deforestation and water pollution player, it directly links the sector to the loss of many species that relied on the wasted land and water. Humans are replacing natural resident species by millions of farmed animals. In fact, livestock already accounts for 20% of the total terrestrial animal life and 30% of this land used to be natural habitats for wildlife that no longer exist⁸.

3. The need for a healthy and sustainable global diet

Diet has a very close relationship with human health and the environment, especially regarding greenhouse gas emissions and all of the impacts discussed before^{14–17}. Therefore, a global dietary transition represents one of the major challenges humanity may have to face. Tilman et al.¹⁸ estimate that in comparison to 2009, a 2050 global-average per capita income-dependent diet will have 18% less fruit and vegetables, 2.7% less plant protein, 23% more pork and poultry, 31% more ruminant meat, 58% more dairy and egg and 82% more fish and seafood.

On the health side, as described above, nations that are starting to have higher incomes are shifting their traditional diets towards others richer in dairy, eggs, meat, fish and seafood, which can correlate to increased incidence of diseases such as type II diabetes^{19–21}, cardiovascular diseases^{22,23} and cancer²⁴. In China, one of the world's fastest growing economies, the incidence of type II diabetes increased from less than 1% in 1980 to 10% in 2008²⁵, while the whole Asian continent already accounts for 60% of the world's diabetic population. Even though Asian people have ethnic and genetic predisposition for diabetes²⁶, the adoption of western food habits and lifestyle is the major factor responsible for these rapidly increasing numbers²¹. In fact, several randomized clinical trials have already shown that type II diabetes is preventable through an adequate and healthy diet^{27–29}. The magnitude of health benefits associated with a balanced diet and reduction of animal derived products is very clear, as it can be observed in Figure 1. In comparison to conventional omnivorous diet,

Vegetarian (V), Pescatarian (P) and Mediterranean (M) diets considerably reduce the risk of developing different types of diseases and decrease overall mortality (Figure 1).

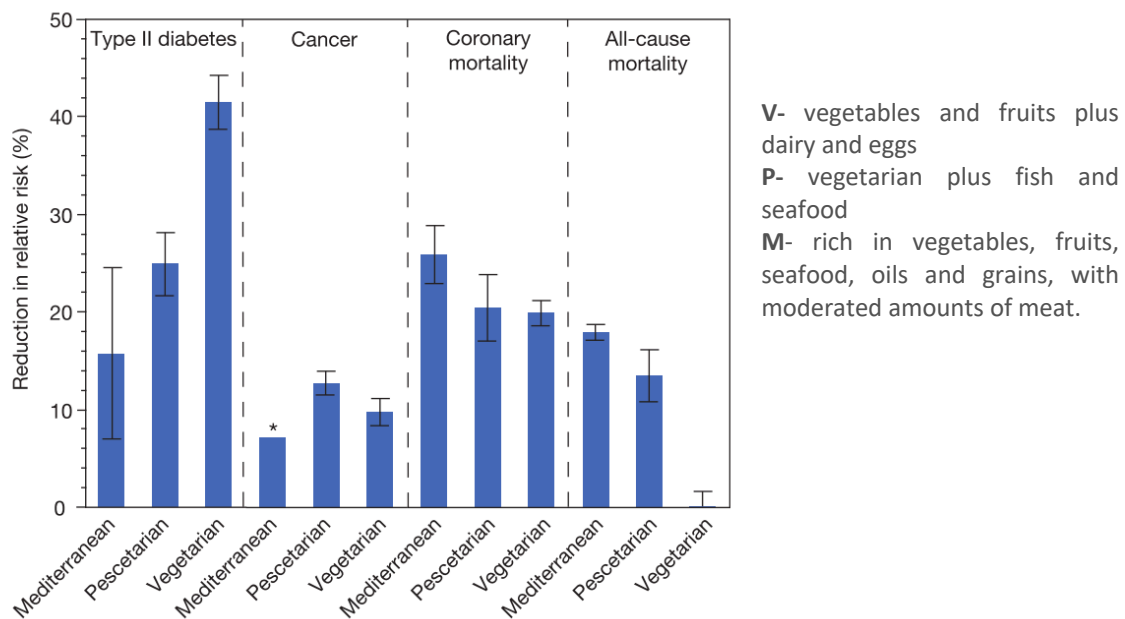


Figure 1 – Diet-dependent relative risk reduction for type II diabetes, cancer, coronary heart disease and all-cause mortality when comparing a conventional omnivorous diet to each alternative diet (M-Mediterranean, P-Pescatarian and V-vegetarian) Adapted from Tilman et al. (Nature, 2014)¹⁸ with permission from the publisher.

On the other hand, foodborne related illnesses are a big concern for health authorities, since animals often live in poor sanitary conditions, being directly slaughtered, which means their meat gets in direct contact with all sorts of pathogens. With data collected between 1998 and 2008, the Center for Disease Control and Prevention (CDC) in the USA attributes more than 26.000 hospitalizations (45.5% of all foodborne illnesses related hospitalizations) each year due to land animal products consumption, with more than 600 deaths per year (43.3% of all foodborne related deaths)³⁰. In fact, a recent report from the US Public Interest Research Group (PIRG) Education Fund³¹ confirms that, between 2013 and 2018, the most hazardous meat and poultry food recalls increased 83%. Unfortunately, the farming industry is not addressing these problems properly. Nowadays, even if this is not openly discussed, it is known that meat producers use chemicals such as ammonia to “clean” the meat, prior to packaging and shipping³². But even these inadequate measures fail, with several outbreaks being reported often³³.

Another major crisis healthcare authorities are facing nowadays relates to the antibiotic resistance that several bacteria strains are acquiring³⁴, not only due to excessive and poorly

controlled use of antibiotics by people, but mainly by the livestock industries. In some countries, such as the USA, almost 80% of all antibiotics are used by the livestock industry, with the World Health Organization (WHO) advising farmers to stop routinely using them³⁵. This further supports that it is imperative to find safer and healthier ways of producing and processing meat and other animal derived products.

Apart from their effect on human health, diets must be sustainable and minimize environmental damage. If consumption of animal-derived products continues to increase in an income-dependent manner, as described, per capita dietary GHG emissions from crops and livestock will increase 32% from 2009 to 2050. Significantly, a Mediterranean diet could reduce those emissions in 30%, a pescatarian diet in 45% and a vegetarian diet could lead to an impressive 55% reduction¹⁸ (Figure 2). Regarding land use, income-dependent diets require, on average, 540 million hectares more than the three alternative diets presented¹⁸. Taking into account all the factors discussed, it is possible to find alternative diets that can significantly and simultaneously improve human and planetary health. In this regard, vegetarianism looks as the most environmental (and animal) friendly alternative. It may eventually also represent a healthy option, but only if balanced correctly, by leaving out foods rich in refined sugars, refined fats, alcohol and oils, also known as “empty calories”.

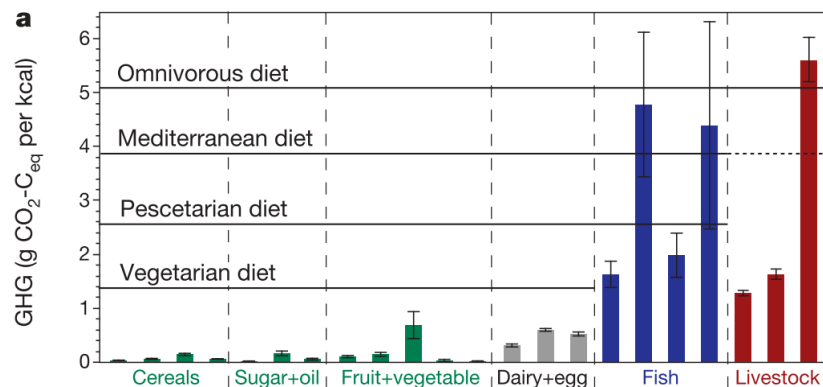


Figure 2 -Lifecycle greenhouse gas (GHG) emissions of CO₂-C_{eq} grams per kilocalorie (kcal) for 22 food products categorized in cereals, sugar + oil, fruit + vegetables, dairy + egg, fish and livestock. Horizontal divisions indicate each diet's threshold. Adapted from Tilman et al (Nature, 2014)¹⁸ with permission of the publisher.

Taking into account the impact of animal farming on the environment, on human health and on the animals' welfare, reducing meat and animal products consumption should be imperative. This could be done by adopting a vegetarian (or even vegan) diet, or at least a “flexitarian/reducetarian” diet. However, the problem relies on how hard it is to make billions

of people change their diets, especially when people don't usually think about long-term consequences. People hardly change their habits, and when they do, it's because better solutions come up, and not because of environmental or health issues.

In order to replace animals in the food sector (as previously done in other sectors), an appealing solution is to combine current knowledge, science and technology to establish new and cleaner ways of producing healthier and safer animal protein. In this context, cellular agriculture and cultured meat emerge as promising solutions, and may one day represent to animal farming what cars represent to horses and electricity to whale oil.

4. Cultured Meat – changing the way we produce meat

Cultured meat, also known as clean meat (as for clean energy and technologies) or cell-based meat, is animal-derived meat, except it is not grown inside the animal. The difference to conventional slaughter meat is that cultured meat uses cell culture and tissue engineering techniques to grow animal cells in a bioreactor, in order to produce the muscle tissue that we would normally harvest from an animal. In essence, the process is no different than brewing beer or making yogurt, with yeast cells being replaced with animal muscle cells, giving rise to a new field: Cellular Agriculture. The process by which cultured meat is produced is schematized in Figure 3, being divided into 4 main phases. The first one comprises cell isolation from an animal tissue biopsy. This can be used to either isolate adult muscle stem cells, so called satellite cells (SC)³⁶, or embryonic stem cells (ESC)^{36,37}. Other somatic adult cells can also be used, reverted to induced pluripotent stem cells (iPSC) and differentiated into myoblasts³⁸. After isolating and selecting the cells of interest, cells must be expanded in a nutrient rich medium, at very large scale, that is why it is crucial to use cell sources with extremely high proliferation capacity or to establish reliable myoblast cell lines.

In this context, ESC would be an attractive cell source, as they hold unlimited self-renewal potential. However, they need special cues to differentiate into muscle cells³⁹, and the chances of accumulating mutations increases after some generations. Also, so far, no bovine, porcine, ovine or of any fish origin ESC lines have been established and, last but not least, they would also carry ethical concerns, which is not aligned with the ideals of cultured meat. On

the other hand, iPSCs are a promising source, since they also hold essentially unlimited self-renewal capacity and don't rise ethical concerns, as opposed to ESC⁴⁰; however, while some studies with porcine- and murine-derived iPSCs^{38,41} showed some encouraging results, further research and development are needed, and better protocols should be established, including for other species such as bovine or fish. Currently, satellite cells are considered as the more adequate source, due to a number of reasons: can be easily isolated from harmless biopsies, are already committed to form muscle tissue, and have the ability to form muscle fibers without specific differentiation cues or manipulation. However, in culture, satellite cells will eventually lose stemness and some strategies are already being explored for maintaining their stem cell state for longer culture periods.³⁶

The second and third phases of the process comprise, respectively, tissue biofabrication and cell differentiation to form muscle tissue. At this stage, cells cultured in 2D monolayers, in microcarriers or as cell aggregates⁴² are normally transferred to an edible and/or degradable animal-free 3D scaffold and cultured in low-serum medium. In order to self-organize into muscle fibers, boosted by protein synthesis, cells need to be anchored to a rigid substrate and sense tension⁴³, one of the most important stimulus for muscle formation. Apart from this, other mechanical stimuli⁴⁴ such as stretching and external electrical^{45,46} stimulation also represent important cues for inducing proper and increased muscle production. Before the fourth and final phase of the process, and depending on the final purpose, a perfusion system⁴⁷ may be needed in the biofabrication phase, if the goal is to produce structured and thick meat products, such as steaks. The aim is to guarantee that cells in the core of the construct have sufficient access to nutrients and oxygen, as the tissue is growing. On the other hand, if ground and processed meat are the goal, biofabrication and perfusion stages might be skipped, and unstructured muscle fibers may be assembled and processed, along with other ingredients, into burgers, meatballs and sausages.

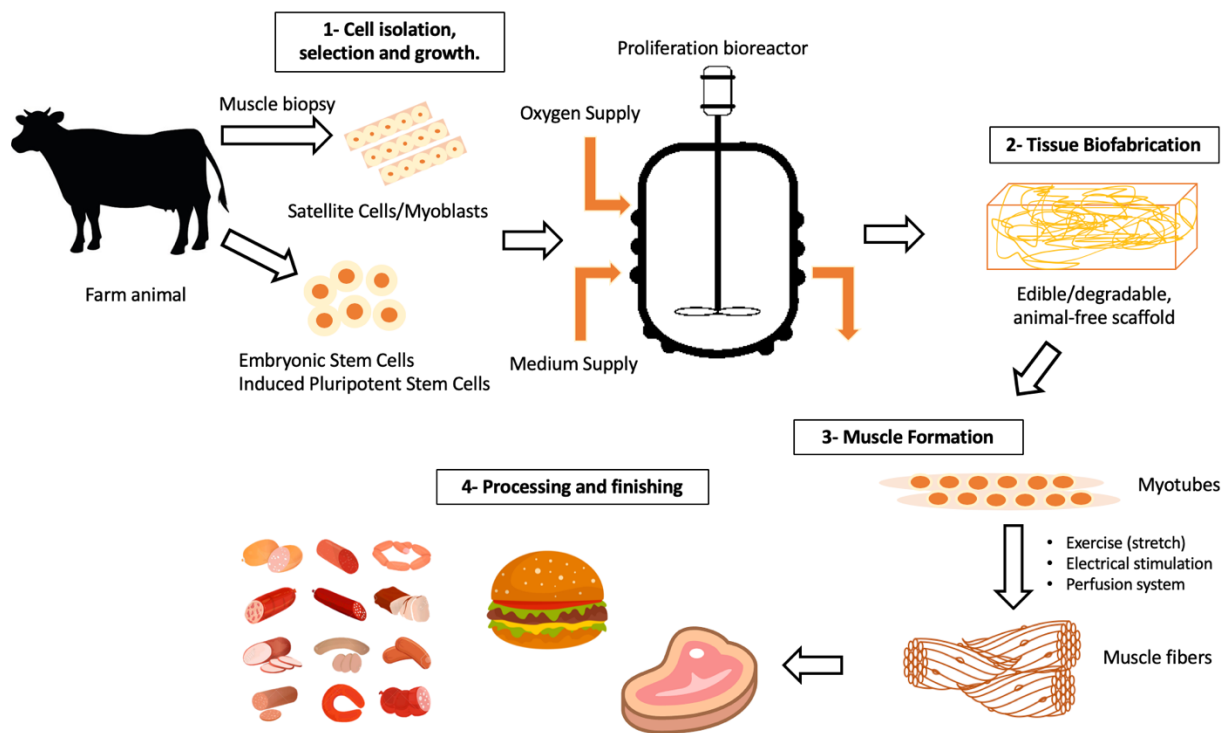


Figure 3 - Cultured meat production overview divided in four main phases: 1- Cell isolation, selection and growth; 2- Tissue Biofabrication; 3- Muscle Formation; 4- Processing and finishing.

While the cellular agriculture field is still in its infancy, it shares a lot of knowledge, techniques and challenges with conventional tissue engineering (TE) strategies for biomedical applications. Both make use of cell culture techniques, scaffolds, biofabrication techniques and bioreactors, with the same main goal: recreate proper 3D environments to produce and assemble native-like tissues *in vitro*. In both cases, advanced scaffolds can be used to promote controlled cell-cell and cell-extracellular matrix (ECM) interactions, and 3D bioprinting has emerged as a powerful biofabrication technique⁴⁸. On the other hand, both are still heavily relying on the use fetal bovine serum (FBS) for cell expansion, which poses numerous limitations, at ethical, safety and economical levels⁴⁹. In contrast to TE for clinical application, in which the tissue must remain alive and functional for successful host integration, cellular agriculture for food production has less strict biological requirements. Cells must proliferate for muscle tissue to grow, but the construct is then processed, cooked and eaten. However, cellular agriculture is still years behind biomedical TE, as it faces other big challenges mainly related to scaling up production at very low and competitive costs. The main issues and challenges related to cultured meat production are described in detail in the next chapter.

The impact of conventional animal farming industries on our health, on the planet and on animals has been described above, but the question that arises is: can cultured meat solve all these problems? Regarding human health, cellular agriculture offers: the possibility to reduce antibiotic usage, a cleaner and safer product and the flexibility to tailor its composition. Produced in a clean, controlled and safe environment, cultured meat does not need antibiotics as livestock does, thus helping fight the antibiotic resistance crisis we face. Furthermore, bacterial contaminations are also less likely to happen and cause foodborne illnesses. On the other hand, the technology's advantage of producing meat from a bottom-up approach (starting with the cells) allows a complete control over meat composition, i.e. include less amounts of saturated fat and/or include healthier ones such as omega-3, in order to reduce the risk of cardiovascular and other diseases.

While it is difficult to deny the health and ethical benefits of cultured meat, several studies are cautious in making definitive claims regarding environmental impact at this stage. Earliest studies, based on life-cycle assessments (LCA)⁵⁰, reported very promising results showing that, in comparison to conventional meat, cultured meat could use up to 45% less energy, release up to 96% less GHG emissions, use up to 99% less land and up to 96% less water. Such values were then updated to more realistic ones in 2014⁵¹. However, as the field is recent and associated technologies are still under early stage development, all predictions at this moment are highly speculative. Another LCA study⁵² reported that cultured meat might, indeed, be better in terms of saving precious resources such as land and water, but a trade-off could exist in energy requirements, when compared to cattle production. Moreover, a very recent work⁵³ conveyed a different approach, taking into account 4 different cultured meat GHG footprints and 3 different cattle beef production systems, under 3 different consumption behaviors, for a 1000 years window. Results shown that the most optimistic cultured meat models outperform the best beef cattle systems, but the gap between them narrows in the long-term, because cultured meat heavily relies on decarbonized energy generation. In conclusion, it is too soon to make solid predictions based on these hypothetical studies, as real data from cultured meat production at effective large scale is still not available.

5. State of the art and challenges ahead

In vivo, skeletal muscle can be formed by three different processes: embryonic myogenesis, adult skeletal myogenesis and muscle regeneration upon trauma⁵⁴. Typically, tissue engineered meat production tries to mimic the regeneration pathway, using satellite cells (muscle stem cells). Upon injury, these cells become activated, proliferating and differentiating into myoblasts, which then fuse into myocytes to form myotubes, the main components of muscle fibers⁵⁵. The ultimate goal in cellular agriculture is to mimic the *in vivo* microenvironment, in order to promote formation of skeletal muscle comparable to native tissue.

Since Prof. Mark Post from the University of Maastricht presented the world's first cultured meat burger back in 2013⁵⁶, funded by Google's co-founder Sergey Brin, a significant number of startup companies have emerged. At that time, Mark Post and his team used FBS supplemented media to grow bovine cells in dozens of multi-layered cell culture flasks, in a very laborious setting (Figure 4), which explains why its cost arose to around 250.000€⁵⁷.



Figure 4 - 10-layer cell culture flasks used to make the world's first cultured burger from Mark Post's laboratory.

Fortunately, along the past 6 years the field advanced, and the cost of producing clean meat has decreased. Mark Post has founded the spinoff MosaMeat to accelerate his team's developments. In 2016, US based start-up company Memphis Meats presented the world's

first meatball⁵⁸, beef fajita, chicken and duck cultured food products. During 2017, plant-based startup company JUST (former Hampton Creek) opened up a cellular agriculture department, being currently one of the best positioned players to bring cultured chicken nuggets to the market⁵⁹. In 2018, another US startup, New Age Meats, hosted a tasting experience for their cultured pork sausages⁶⁰. More recently, Israeli based startup Aleph Farms presented the world's first steak strip based on a texturized soy scaffold⁶¹. As of June 2019, there are around 43 cellular agriculture startups (and more in stealth mode) around the world, tackling not only meat production, but also other animal derived products such as fish, fat tissue, dairy, eggs, silk and leather. Most of the work being done by these companies is not publicly shared, as they are still in a developing stage and heavily rely on their own intellectual property (IP) to become competitive in the future. However, some patents are starting to be published, shading some light into the plans and main challenges each team is tackling.

As pointed out, one of the major challenges the field faces is cost reduction. The excessive costs mainly derive from the use of substantial amounts of culture media and FBS, needed to culture huge numbers of cells. For years, attempts are being made to achieve FBS-free media formulations that do not compromise cell proliferation and successfully replace FBS⁶²⁻⁶⁴, which by its own also raises multiple concerns. However, to make cultured meat cost competitive, further efforts still need to be done to find cheap, animal-free and highly available formulations of culture media, allowing thousands of liters to be produced at a low cost. One out-of-the-box, yet controversial, solution has been recently revealed by a patent submitted by Memphis Meats⁶⁵. It makes use of the CRISPR-Cas9 system to genetically modify cells, inactivating the HIPPO pathway, and improving their growth in serum-free media. This modification also shifts muscle cells towards an anchorage independent phenotype, further allowing their culture in suspension for higher biomasses production.

With a reliable cell source, either a primary culture or a cell line, and a cheap and animal free media, the path is strongly paved towards commercialization. However, in order to scale up production and achieve cost reduction at low carbon footprint, the development of industrial proliferation bioreactors in the order of the 5000 liters capacity is crucial⁶⁶. The pharmaceutical industry is well used to the bioprocessing of large quantities of biological products⁶⁷, but not at the high-scale cultured meat needs (to achieve 1kg of protein from muscle cells, around 8×10^{12} cells are needed⁶⁶). Hollow fibers, fluidized bed and microcarriers

systems based bioreactors are current options being explored^{66,68}. The scenario gets even more complicated because differentiation of the cells is also needed, usually accomplished by decreasing serum concentration from 10-20% to 0.5-2% and by replacing FBS by horse serum (HS)^{69,70}.

Once solved the three challenges mentioned above, ground meat products such as burgers, meatballs, sausages and nuggets should be ready to enter the market. Yet, to achieve a native tissue-like piece of muscle (e.g. steak), a highly structured and organized tissue, the use of adequate 3D scaffolds is required. The development of animal-free, scalable and optimal guiding scaffolds for muscle growth represents another huge challenge cellular agriculture has to overcome. Such scaffolds must eventually be perfused to ensure cell survival and function, in order to surpass nutrients and oxygen diffusion limits^{71,72} and produce thick tissues. In tissue engineering for clinical applications, recent efforts are being devoted to artificial vascularization approaches. In particular, 3D bioprinting technology is being explored to build perfusable scaffolds, where channeled networks are obtained upon removal of sacrificial bioinks^{73,74}. Yet, such strategies are difficult to apply in a cheap, easy and scalable way, which is essential as already discussed.

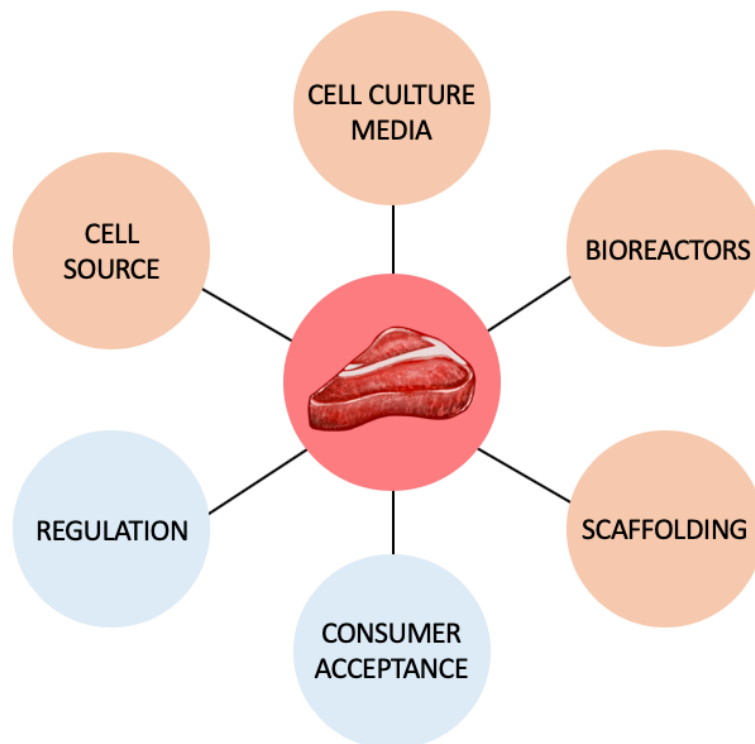


Figure 5 - Technical (orange) and non-technical (blue) challenges around cultured meat and cellular agriculture.

Apart from all those technical challenges, the industry also faces roadblocks related with regulation and consumer acceptance. Regulatory affairs are always a very tricky subject and will not be fully covered here. Briefly, cultured meat regulation in the European Union (EU) should fall under the Novel Food Legislation, whether in the USA, the US Department of Agriculture (USDA) and the Food and Drug Administration (FDA) recently announced a joint formal agreement to regulate cell-cultured food products⁷⁵. When it comes to consumer acceptance, various studies, surveys and focus groups have been done⁷⁶, where “disgust” and the sense of “unnatural” commonly arise as negative public perceptions and reactions⁷⁷. However, this same last study⁷⁷ found that the more people learned about cultured meat, the more supportive they became. In the end, everything will rely on consumer education and awareness. Still, for most consumers, the decision will ultimately be based on the price, taste, safety and convenience of cultured meat, as compared to conventional meat.

Motivation & Goals

As described above, the cellular agriculture field still faces many challenges, but also represents unique opportunities. In this work, to address some of the current needs, a scalable solution for creating thick (>5 mm) bioengineered tissue constructs is proposed, based on the use of a perfusable device for dynamic culture in a bioreactor. In addition, some experiments were carried out aiming at establishing an adequate source and isolation/culture protocol for obtaining bovine muscle cells, and also testing the adequacy of a non-animal derived scaffold.

In summary, the main goals of this work were:

1. Design and fabricate a 3D printed device for the growth and maintenance of thick engineered tissues, under dynamic conditions, containing milli-channels for culture media perfusion.
2. Bioengineer viable muscle tissue-like thick 3D constructs, by culturing myoblasts in an animal-free scaffold, using the perfusable device.
3. Isolate, purify, expand and characterize bovine whole muscle cell population and satellite cells from fresh muscle tissue.

Materials & Methods

1. Device design, prototyping and testing

1.1 Design and 3D printing of the device

All versions of the platform (perfusable device and accessories) were designed using AutoCAD software Fusion 360 from Autodesk® for MacOS, and were 3D printed in a Stratasys® Objet Eden260V at a 16 µm layer height. The material used was VeroGray™, a proprietary polymeric resin based on Exo-1,7,7-trimethylbicyclo[2.2.1]hept-2-yl acrylate and 4-(1-Oxo-2-propenyl)-morpholine. In order to increase strength of the platform components, from version 4.1 onwards, each device was cured under UV light for 30 min, to increase polymerization of the resin. A gentle waterjet was used to remove the bulk of water-soluble support material, whereas the tip of a sewing needle and a paperclip were used to carefully remove any residual material present in between the channels and pores.

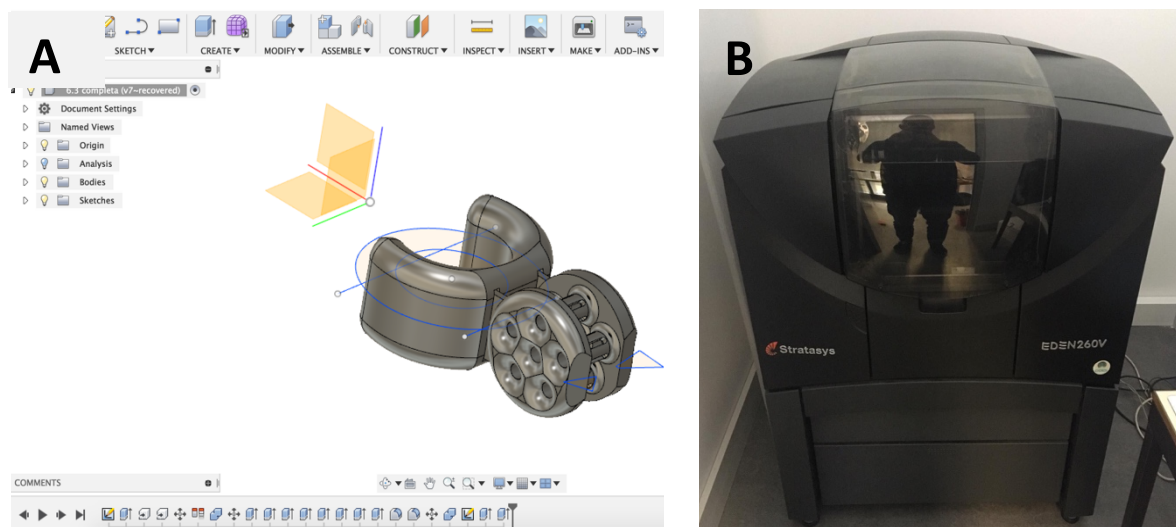


Figure 6 – A) 3D design modelling of the device using Fusion 360 from Autodesk®. B) Stratasys® Objet EDEN260V stereolithography 3D printer.

1.2 Alginate preparation

Food grade sodium alginate powder with a high content of guluronic acid units (Protanal LF20/40, ca. 70% guluronic-to-mannuronic acid monomers) was a kind gift from FMC Biopolymer A.S. (Drammen, Norway) and was used as scaffolding biomaterial. When indicated, covalent grafting of the oligopeptidic sequence of (Glycine)₄-Arginine-Glycine-

Aspartic acid-Serine-Proline (abbreviated as RGD; Peptide International, USA) was carried out using aqueous carbodiimide chemistry. The reaction was carried out as described by Mooney *et al*⁷⁸ using 16 mg of peptide per gram of alginate. When sterility was required, a 0.5 wt% solution of alginate in dH₂O was prepared, filtered through a 0.2 µm filter, freeze-dried and then reconstituted at the desired concentration.

1.3 Alginate hydrogel formation and casting on the device

Hydrogel formation was carried out using an internal gelation strategy as previously described by Fonseca *et al.*⁷⁹. Briefly, alginate gel precursor solutions at 1.7 wt% (for a final concentration of 1 wt%) and 2.7 wt% (for a final concentration of 2 wt%) in 0.9% w/v NaCl were mixed with an aqueous suspension of calcium carbonate (CaCO₃, Fluka) until homogenization. Release of the calcium ions and gelation was triggered by adding a fresh solution of gluconic delta-lactone (GDL, Sigma). The molar ratio of CaCO₃/GDL used was 0.125. Hydrogel formation was allowed to proceed under standard incubator conditions (37 °C, 5% CO₂) for approximately 30 to 45 min.

In order to form alginate hydrogels with the shape of the device, a cast (made of the same material as the device) was used either to produce bulk alginate cylinders (V=1 mL) or perfused alginate cylinders (V=750 µL). Alginate precursor solution was allowed to crosslink inside these casts. After gelation, the cylinders were used to perform the food coloring perfusion experiments and for 3D cell culture. Static conditions were tested by immersing hydrogel discs in 100 mL flask (Figure 7A), and dynamic conditions were tested by mounting the device in a spinner-flask under magnetic stirring at 100 rpm (Figure 7B).

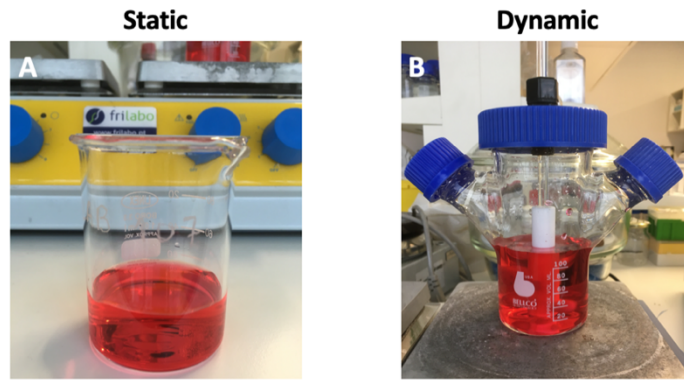


Figure 7 - A) Static conditions setup using a 100mL flask. B) Dynamic conditions setup using a spinner-flask and a magnetic plate at 100 rpm.

1.4 C2C12 mouse skeletal muscle cell culture

C2C12 mouse skeletal myoblasts cell line was a kind gift from Dr. Pedro Moreno (i3S) and was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with penicillin/streptomycin (P/S) 1% v/v (Gibco), supplemented with 15% v/v FBS (Bio West) – hereafter referred as complete DMEM. Myoblasts were maintained under standard incubation conditions (37 °C, 5% CO₂), and passaged when reaching sub confluent (70-80%) culture, every two days usually at 1:2 or 1:3 ratios. Briefly, medium was discarded, and cells were incubated with 0.03 mL/cm² of 0.25 M trypsin used 2.5 min at 37 °C to detach cells.

1.5 Cytotoxicity assay of the polymeric resin

To evaluate the cytotoxicity of the polymeric resin (VeroGray™) an indirect cytotoxicity assay (extract assay) was used. The device was mounted inside a spinner flask with DMEM supplement with 1% v/v P/S, and incubated at 37°C under 100 rpm rotation, for 1 and 7 days, after which conditioned medium (CM) was collected. C2C12 cells were seeded at 10.000 cells per well in a 24-well plate and were cultured with either fresh medium, 1-day CM or 7-days CM. Each medium was supplemented with 15% FBS, just before being added to cells, and conditions were assayed in triplicates. Metabolic activity of the cells was assessed at days 0, 1, 3 and 7, using the resazurin assay⁸⁰. Briefly, after each time point, the medium of each well was removed and replaced with 500 µL of 20% resazurin in DMEM (1% v/v P/S + 15% v/v FBS). Cells were incubated for 2 h at 37 °C and 200 µL/well were transferred into a black clear bottom 96-well plate. Fluorescence, resulting from resazurin reduction to the fluorescent

compound resofurin, were measured using a Biotek™ Synergy™ MX fluorometer at 530 nm excitation and 590 nm emission, and expressed as relative fluorescence units (RFU).

2. Device validation

2.1 3D culture of C2C12 myoblasts in alginate hydrogels

C2C12 myoblasts were embedded at 14×10^6 cells per mL of alginate hydrogel (final concentration of 2 wt%) using the previously described internal gelation method. After casting the discs, using either the “bulk” mold or the “holes” mold (see Fig. 8), these were either transferred to a 50 mL ventilated tube (static conditions) or to the device’s version 6.3 and placed inside a spinner flask under rotation at 100 rpm (dynamic conditions) with complete DMEM. All samples were maintained under standard culture conditions and analyzed at day 0 (processed within 1h-1.5h after casting) and at day3.

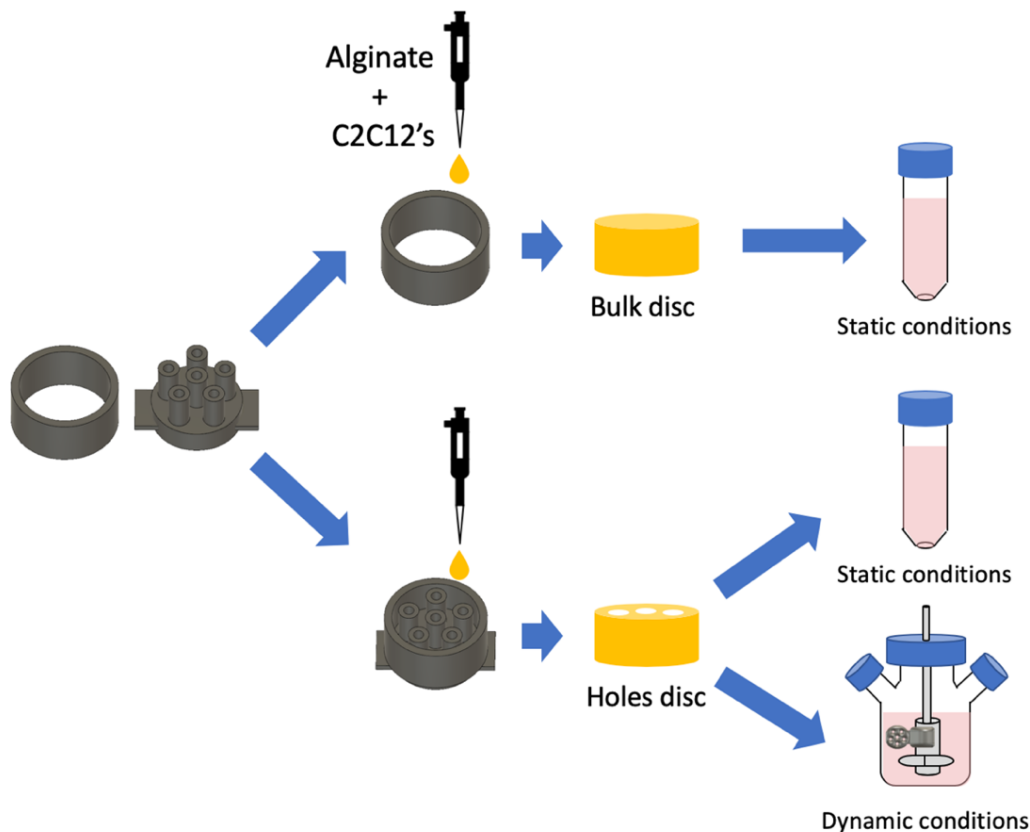


Figure 8 - Casting and cultivation of alginate+ C2C12's discs in static and dynamic conditions.

2.2 Live/dead assay

In order to analyze the viability of cells after culture within the alginate hydrogel-based 3D matrix, central transversal 2 mm thick slices were obtained from the original discs with a scalpel. Stock solutions of Ethidium Homodimer-1 (2 mM in DMSO:ddH₂O 1:4, Invitrogen) and Calcein (1 mg/mL in DMSO, Invitrogen) were prepared beforehand. Each slice was then washed with FBS-free DMEM without phenol red (Gibco) and incubated in 1.5 mL of this medium with 0.25% ethidium homodimer stock solution and 0.20% calcein stock solution at 37 °C for 45 min. Slices were washed and maintained in fresh DMEM without phenol red, and immediately analyzed by confocal laser scanning microscopy (CLSM) using a Leica TCS SP5 microscope. “Green” and “red” areas were quantified using 5 different and randomly chosen tiles and measuring the signal mean intensity for each channel using Fiji. The average of all 5 tiles was used and the percentage of “green” vs. “red” signal calculated over the total signal mean intensity.

2.3 Metabolic activity assay

After culture, C2C12 myoblasts were recovered from the alginate hydrogels discs by mincing the discs with a scalpel and adding 5 mL/disc of trypsin/EDTA solution for 7 min at 37 °C, for hydrogel dissolution and cell release. After inhibiting trypsin activity with complete medium, the mixture was centrifuged at 1100 rpm for 5 min. The supernatant was discarded, and the pellet resuspended in 7 mL of 20% resazurin in complete DMEM. The total amount of cells recovered from each disc was seeded in a 6-well plate and incubated for 1.5h at 37 °C, 5% CO₂. Wells with resazurin solution but no cells were used as controls. After incubation, 600 µL/well were collected, centrifuged to pellet down any cells and transferred in triplicate (200 µL) to a black clear bottom 96-well plate. Fluorescence was measured as described in section 1.5.

3. Bovine muscle cells isolation and characterization

3.1 Establishment of primary cultures of bovine muscle

Bovine muscle tissue samples were a gift from a local slaughterhouse. The isolation procedure was adapted from Ding *et al.*³⁶ and Hindi *et al.*⁸¹, with some modifications. Bovine muscle tissue (50 g) from the neck was freshly harvested from 1-year-old cattle and immediately transported to the lab on ice. Samples were washed with ethanol 70% prior to mechanical dissection in 2-5 mm pieces. Then, the tissue was digested with collagenase type IA (Sigma Aldrich, 0.2% w/v) in DMEM (Gibco) supplemented with 1% v/v P/S (Gibco) (Digestion Medium) for 1.5 h at 37 °C, under continuous agitation and vortexing every 15 min. A volume of 30 mL of digestion medium per 5 g of tissue was used. After digestion (no intact muscle pieces visible), 20% v/v FBS was added to inhibit collagenase's activity. The mixture was centrifuged at 80 g for 3 min to pellet down any remaining undigested tissue and the supernatant collected as a cell suspension. This suspension was centrifuged at 1000 g for 5 min and the supernatant discarded. Isolated cells in the pellet were resuspended in sterile PBS 1X and washed twice by centrifugation at 1000 g for 5 min plus resuspension in PBS 1X. After washing, the cell pellet was resuspended in 10 mL of DMEM with 15% v/v FBS and 2% v/v P/S (Isolation Media) using a sterile 10 mL serological pipette and the tissue was pipetted up and down 20-30 times to ensure that most cells were released from the tissue. Next, the cell suspension was passed through a pre-wetted 70 µm cell strainer into a 50 mL tube. The same procedure was repeated with a 40 µm cell strainer, allowing most of the tissue debris to be removed. The final cell suspension was centrifuged at 1000 g for 5min and the pellet resuspended and incubated in 5 mL Red Blood Cells (RBC) Lysis Buffer (10 mM Tris, 150 mM NH₄Cl, pH=7.4) at 37 °C for 7 min. After, 10 mL of sterile PBS 1X was added and cells were washed twice. The RBCs-free pellet was resuspended in 10 mL of Nutrient Mixture F-10 Ham (Gibco) supplemented with 17.5% v/v FBS and 1.5% v/v P/S (Culture Medium). The total number of cells was counted in a Neubauer's chamber. Finally, cells were either frozen in F-10 + 40% v/v FBS + 10% v/v DMSO (Freezing Media), directly plated onto 0.05% v/v type I collagen (in PBS) (Sigma Aldrich) pre-coated 6-well plates or processed for fluorescence-activated cell sorting (FACS).

3.2 FACS sorting of bovine satellite cells

For antibody testing and flow cytometry analysis, cell suspensions were centrifuged at 1200 rpm for 7 min and resuspended in 5 mL of FACS buffer (0.5% w/v BSA, 0.01% w/v sodium azide in PBS), distributed by five 15mL tubes and pelleted down again. After centrifugation, cells were resuspended in reminiscent buffer (50-100 μ L) and incubated with APC anti-human CD29 antibody (1:10, BioLegend, Cat#303008), PE Mouse anti-human CD56 (1:10, BD, Cat#555516), FITC Mouse anti-human CD31 (1:10, BD, Cat#555445), FITC Mouse anti-bovine CD45 (1:10, Bio-Rad, Cat#MCA832F) for 30 min on ice protected from light.

For fluorescence-activated cell sorting (FACS) of CD31⁻ CD45⁻ CD29⁺ CD56⁺ viable cells (satellite cell population), after staining, cells were washed with 10mL 1X PBS and kept at 4 °C until analyzed. Cell sorting was performed on a BD FACS Aria II Inc cell sorter, using 488, 561 and 633nm lasers. Unstained and monolabeled cells were used to define FACS gating parameters.

3.3 Bovine muscle cell culture

Whole muscle cell population (unsorted cells, used as controls) and isolated satellite cells were cultured in Nutrient Mixture F-10 Ham (Gibco) supplemented with 17.5% v/v FBS, 1.5% v/v P/S and 10 ng/mL FGF (37 °C, 5% CO₂). Cells were always maintained until reaching sub-confluent cultures, being passaged at around 60-70% confluency, the same way as described for the C2C12 cell line. Bovine satellite cells differentiation was induced at around 90% confluency, by culturing cells in DMEM + 2% v/v FBS (Bio West) Cell morphology was monitored along time using an inverted bright-field microscope (Olympus CK X 41).

3.4 Metabolic activity assessment

Metabolic activity of sorted CD31⁻ CD45⁻ CD29⁺ CD56⁺ cells was assessed at days 3, 7, 10 and 15 post- sorting. Briefly, cells were seeded at 5.000/well (24-well plate) after isolation. At each time-point, the medium was removed and replaced with 500 μ L of 20% resazurin in Culture Medium. Cells were incubated for 1.5 h at 37 °C and 200 μ L/well were transferred to a black clear bottom 96-well plate. Fluorescence was measured as described in section 1.5.

Each well was then washed with F-10 medium to remove resazurin leftovers and cultured in regular supplemented F-10 medium (Culture Medium).

3.5 Immunofluorescence analysis of cultured bovine cells

Cultured sorted (CD31⁻ CD45⁻ CD29⁺ CD56⁺) cells and unsorted cells were fixed in 4% PFA (in PBS) for 20 min at room temperature (RT, ~21°C). Cells were then washed with PBS 1X, permeabilized in 0.5% v/v Triton X-100 (in PBS) for 15 min and blocked in 1.5% w/v BSA (Sigma Aldrich) for 1 h. Samples were then incubated overnight at 4 °C in 1% w/v BSA with anti-mouse PAX7 (1:10, Developmental Studies Hybridoma Bank, Cat#PAX7). After washing 3x for 5 min with 1.5% w/v BSA, cells were incubated with Alexa 488 anti-mouse (1:600, Invitrogen, Cat #A-11059) and DAPI (1:500, Vector Laboratories, Inc) for 1 h at RT, protected from light. Then, cells were washed 3x for 5 min with PBS 1X and mounted with VECTASHIELD Antifade® mounting medium. Images were obtained using IN Cell Analyzer 2000 (GE). For PAX7⁺ cells identification, a threshold of signal intensity was defined after histogram plotting of the mean intensity signal of PAX7 for each cell. DAPI staining was used to identify cell's nuclei. PAX7⁺ cells were counted using 5 different and randomly chosen fields for each condition.

Results and Discussion

1. Device design, prototyping and testing

1.1. Optimization of the perfusable device

In order to establish and maintain nutrients/oxygen supply and metabolites removal throughout the entire thickness of an engineered tissue, one option is to provide continuous flow perfusion across the 3D structure during culture. In this context, one of the main goals of this project was to fabricate a perfusable device that would provide this. Along the project, different prototypes have been designed, fabricated and tested, until achieving an optimized final version, which was then validated.

The original prototype was made of paper (see Table S1, Annex A), which then translated into the design of version 1.0. It consisted of a cubic box with 6x6 vertical porous channels incorporated inside. The idea was to couple the device to a peristaltic pump for perfusion, and so, it incorporated a tube adapter in the back. This would presumably ensure medium circulation through the channels and its delivery through the pores, in order to feed cells within a scaffold mounted on the device. After analyzing the feasibility of using a feeding system using a peristaltic pump, we concluded that the setting would become too complex and difficult to scale-up. Furthermore, after printing the first prototype of version 1.0, we realized that the initially designed channels were too thin (1 mm of internal diameter) and too close to each other (0.6 mm), making it almost impossible to introduce a cell-laden scaffold inside the device. It would also be extremely difficult to maintain the integrity of the engineered tissue when attempting to remove it from the device after culture. On top of this, the pore size was also too small (1 mm x 1 mm) to allow an efficient delivery of medium to the scaffold-embedded cells (see Figure 9A).

After rethinking the design of the device and deciding to change the perfusion system, version 2.0 was created and paved the way for all versions created onwards. Version 2.0 uses an adaptation ring that allows the device to be connected to the central spindle of a spinner flask, using its rotation to promote medium perfusion through the channels. When the spindle starts to rotate, induced by a magnetic field applied on the magnet present in the bottom, the

device is propelled against the surrounding medium, forcing it to enter/leave the channels, thus promoting perfusion for feeding the cells. This strategy is simpler and also allows the continuous re-use of the medium that is going through the channels. For implementing this approach, the only components needed are a bioreactor with a central spindle (e.g. spinner-flask) that rotates, and an adaptable perfusable device. In version 2.0 the internal channels diameter was also increased to 2 mm and the distance between channels to 2 mm, changing pore shape from squares to circumferences (Figure 9A) and adopting a rectangular shape (with 6 channels) to fit inside the available spinner flasks (Table S1, Annex A). However, we later on realized that a rectangular shape would hold dead zones in the corners, where the medium would not be able to efficiently diffuse to. Therefore, version 3.0 marked the transition to a circular device, composed of a cylindrical box with two components (see Table 2): a lid attached to the ring adapter and the main component with the channels that perfectly fitted the lid.

Table 2 - Main versions of the device's prototyping process.

Version		3.0	4.1	5.1	6.2	6.3	6.4
3D Model							
Description		<u>Cylindric box</u> with 6 hollow and vertical channels with <u>circular pores</u> . Fixed ring adapter for a spinner flask central spindle.	<u>Cylindric box</u> with 6 hollow and vertical channels with <u>elongated pores</u> . Fixed ring adapter for a spinner flask central spindle.	<u>3 components</u> with 6 hollow and vertical channels with elongated pores. Fixed ring adapter for a spinner flask central spindle.	<u>Open device with 1 mm thick channels and elongated pores</u> . <u>Double-support ring adapter</u> for a spinner flask central spindle.	Open device with <u>0.75 mm thick channels and elongated pores</u> . <u>Double-support ring adapter</u> for a spinner flask central spindle.	<u>Full ring scalable holder for 4 devices of version 6.3</u>
Features	Height	5 mm	6 mm	6 mm	6 mm	6 mm	N/A
	Diameter	15 mm	15 mm	15 mm	15 mm	15 mm	N/A
	External channel diameter	3 mm	3 mm	3.5 mm	4 mm	3.5 mm	N/A
	Internal channel diameter	2 mm	2 mm	2 mm	2 mm	2 mm	N/A
	Pore diameter	2 mm	0.7 mm	0.7 mm	0.7 mm	0.7 mm	N/A
	Pore length	N/A	4.35 mm	4.35 mm	4.35 mm	4.35 mm	N/A
	Distance between channels	Max. 1.5 mm Min. 2.3 mm	1.5 mm 2.3 mm	1 mm 1.8 mm	1 mm 1.8 mm	1 mm 1.8 mm	N/A N/A

Note: The maximum distance between channels refers to the distance between each peripheral channel, while the minimum is the distance between the central channels and any peripheral channel.

For better refining the prototype we needed a more practical input, so we started testing the device in a mock situation. By using 1 wt% alginate hydrogel discs as a scaffold and a solution with red food coloring as “medium”, we were able to monitor the diffusion of the red dye into the transparent hydrogel, throughout time. As observed in Figure 10A, and as compared to bulk-discs and holes-discs (static) used as controls, version 3.0 showed very poor diffusion. At the time, we believed this was due to the small size of the circular pores (0.7 mm in diameter), which were not allowing sufficient contact between the hydrogel and the fluid, as compared to the controls where samples were totally submersed in it. This led to another reshaping of the pores, crating longer pores in version 4.0 channels, by fusing the top and the bottom circular pores into a longer one (see Figure 9A). From version 4.0 onwards, pores were always designed with an elongated shape, which increases 2.5-fold the contact area between the fluid going through the channels and the scaffold inside the device. This new feature and its benefits were confirmed, as shown in Figure 10A, by the improvement in red color diffusion, when comparing version 3.0 and 4.0 at any time point.

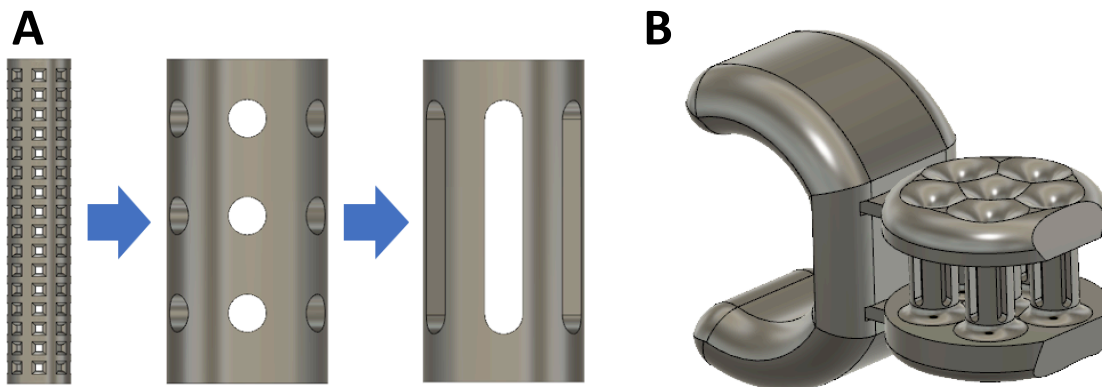


Figure 9 - A) Pore shape evolution from square to circular to elongated, from version 1.0 to 2.0 to 4.0 onwards. B) Final version of the device (v6.3).

However, comparing version 4.0 to the holes-disc (static) control, it was clear that this version was still behind our expectations and there was room for improvement. Apart from this, versions 3.0 and 4.0 presented some technical challenges in terms of manipulation: 1) their channels were too thin and fragile, and would frequently break, as observed in Figure S1, Annex A. 2) the two components were hard to fit into each other because the integrated closed system did not allow to easily match the channels' top and the small incisions on the lid. To solve these issues, version 5.1 (after 5.0) was designed as a 3 components device with an increased external channel diameter (3.5 mm), but the same internal diameter (to provide solid channels), and a consequently decreased distance between the channels (from a

maximum of 2.3 mm to 1.8 mm). The 3 components setting allowed a much easier assembly of the device, whereas the increased thickness of the channels improved their strength, facilitating manipulation without breaking. Furthermore, as the distance between channels decreased, the diffusion of the red dye into the alginate was improved, as observed in Figure 10A.

Yet, the performance of version 5.1, as compared against the static controls, was still below our expectations. We realized that, up to then, we always had tested quite closed devices, where the contact area between sample and surrounding medium was limited to the channels' pores, unlike the controls that were totally immersed/surrounded by medium. While there was a need of increasing the contact area, we could no longer increase the pores' size, as this would risk compromising the integrity of the already fragile channels. Therefore, the solution implemented in all version 6 series (Figure 9B) was based on a new design, with a more open system to maximize contact between the scaffold and the medium. All the walls around the disc were removed, adding an extra of 300 mm² of contact area with the surrounding fluid. This new open design forced us to abandon the ring adapter (which was attached to the walls around the device) and a new double-support ring system was created. The performance of version 6.2 (an improved version of 6.0), as depicted in Figure 10A, shows significantly improved diffusion of the red dye into the alginate. While it appears that the controls present similar levels of coloring, it is important to highlight that this is a visual artifact. In fact, only the periphery, which becomes rapidly colored, is being photographed, masking the real diffusion progression. Looking at pictures of central slices of the bulk control disc, it is possible to see that, despite the surface being colored, the core of the disc is not, even after 1 h and 10 min of immersion (Figure 10 B1.1). This is even more exacerbated in a denser 2 wt% alginate bulk disc (Figure 10 B1.2). The same observation could not be made in the holes-discs control, as they lack a solid core due to the presence of 6 evenly distributed transversal holes. Nevertheless, analyzing the progress of the bulk disc (first row of Fig.10A), one can clearly see the diffusion of the red color coming from the periphery, which is then masked at 40 min, because the top and bottom surfaces get colored, hiding the core from being observed in the picture.

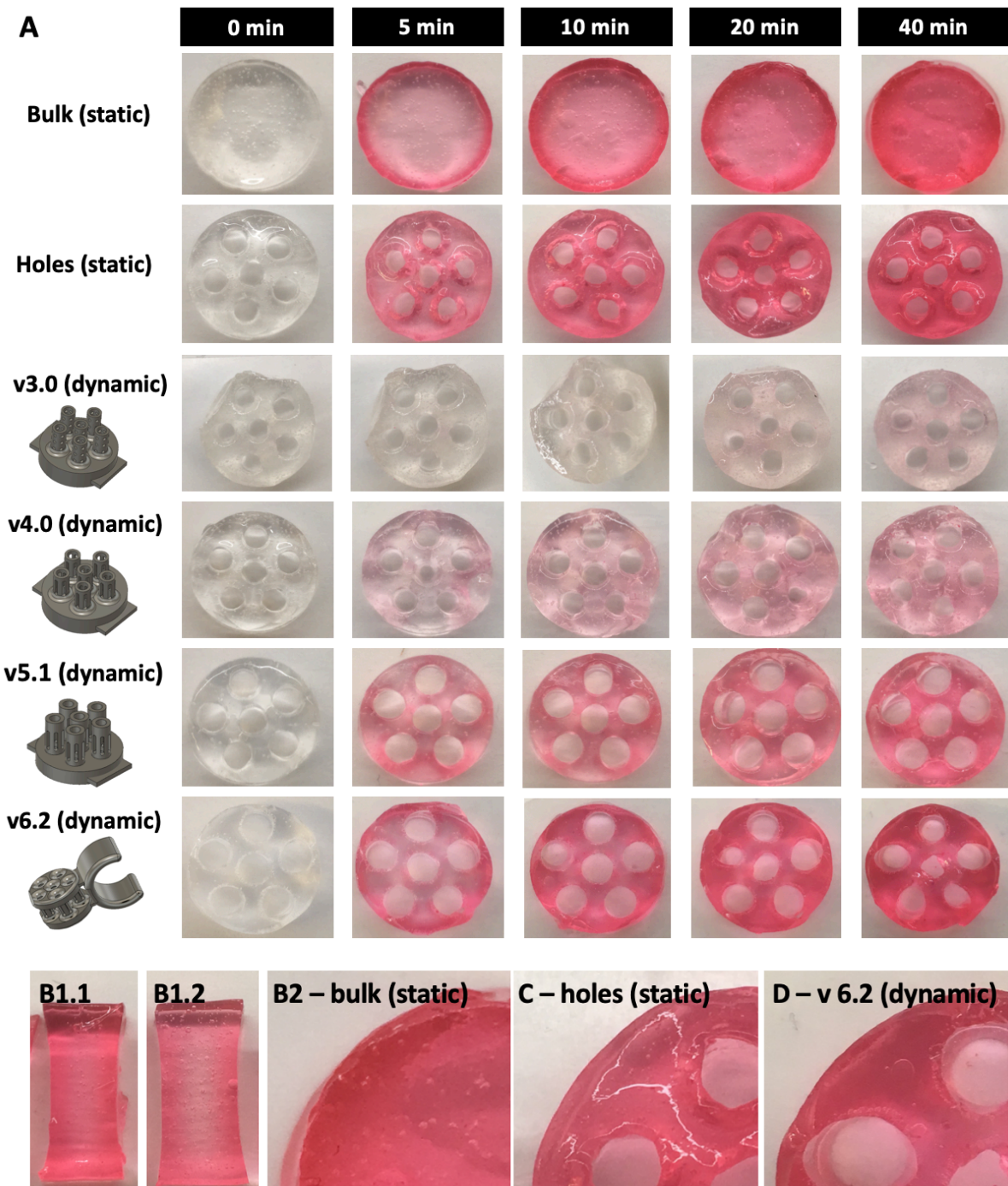


Figure 10 - A) Evolution throughout time of the red food coloring diffusion in the different main versions of the device, including static bulk and holes controls. B1.1) Central slice of a 1 wt% alginate bulk disc cultured in static conditions for 1h and 10min. B1.2) Central slice of a 2 wt% alginate bulk disc cultured in static conditions for 1h and 10min. B2) Zoomed in image of the top left corner of bulk (static) at 40min. C) Zoomed in image of the top left corner of holes (static) at 40min. D) Zoomed in image of the top left corner of v6.2 at 40min.

Still, results obtained with the holes-discs (static) control and the device (version 6.2) were similar. However, one important fact to take into account is that these discs are only 6 mm thick. Although not tested here due to the lack of a bigger bioreactor, we believe this would not happen with larger and thicker samples. For dimensions compatible with a meat-like product, these differences would become much more apparent, and limitations of the static approach would become clear. The fact that the discs are only 6 mm thick means there is only a 6 mm long path the fluid has to go through. In static conditions, diffusion is the main responsible for mass transfer⁷¹ and the distance here is small enough to be assured by the diffusion rates of the fluid, as the food coloring solution is being absorbed by the alginate (which is less concentrated than the outside environment). In fact, this absorption of the red dye water solution was observed by the swelling of the disc throughout time (not shown). On the other hand, due to fact that the device is designed to be scalable, we believe that increasing the channels' length (and consequently the pores') would not compromise the performance shown at this level, because it does not change the device's design and way of working. Nevertheless, this is something that needs to be further assessed at a bigger scale, and with larger/thicker discs.

The final and last version of the device was version 6.3 (Figure 9B), which, although not tested in the red food coloring experiments, presents slightly altered channels thickness (0.75 mm). This last small change was introduced later on, during cell culture experiments, as it showed to provide a better compromise between inter-channels distance (enough to provide sufficient culturable space for the scaffold) and adequate channels strength and integrity for easy manipulation without breaking. Version 6.3 presented an available/culturable volume of 714 mm³ (for mounting the scaffold), which represents 67.3% of the device's volume, with the remaining being occupied by the 6 channels. Also, in order to fit inside the spinner flask and allow free rotation, top and bottom parts of the device had to be trimmed. At that stage, the device presented a functional design, being easy to manipulate, and allowing introduction and removal of the pre-casted disc without damaging it. After being casted in the designed mold, the 6 holes disc is introduced in the channeled component, which is then closed with the top part of the device (see Annex B, Figure S2). When assembled, the components, containing the sample, perfectly align and fit into the double-support ring, which is then attached to the spinner flask (see Annex B, Figure S2). The reverse procedure should be followed for device/sample removal.

For being used at industrial-scale tissue engineering applications, namely for production of large pieces of cultured meat tissue, the device should be easy to scale-up. We believe that this is actually one of the major features of the proposed device, which although not directly approached here, was always taken into account during designing steps. In a scaled-up device, the size would be increasing, along with the number of channels needed to feed the tissue, while at the same time will also be possible to increase thickness, increasing channels length. Version 6.4 further illustrates the scalability of the system (see Table 2), as it would allow higher throughput production of several pieces of meat at the same time, within the same bioreactor. Additionally, several devices could be mounted on-top of each other, to take advantage of the vertical space still available in the flask, thus further increasing even more the throughput. Something important to notice is that this bioreactor is not a proliferation-specific bioreactor, but a biofabrication one. In order to get billions/trillions of cells, other types of bioreactors are more suitable, e.g. suspension cultures or hollow fibers bioreactors. Furthermore, despite some advantageous features, the scaffold used in this work was not the ideal one for cell proliferation and tissue formation, thus cultured meat production. A more suitable scaffolding material would have to be cheap, not require expensive functionalization steps and allow the 3D microenvironment remodeling. Culturing time would then need to be the minimum for cells to organize in the scaffold, remodeling it and producing their own ECM, as well as differentiating to form muscle fibers and a final muscle tissue.

Theoretically, other device shapes could be explored, with adequate channels positioning, allowing to modeling specific cut of meats: adopting a chicken breast or a T-bone steak shape, for example. The holes left by the channels might eventually disappear if the tissue is maintained for a while in culture outside the device, due to natural tissue contraction. Actually, in a larger piece of meat, such small holes (3.5 mm in diameter) would no longer be visually significant. Alternatively, the holes could be filled with additional components, such as fat or aromatic herbs, to improve the flavor of the meat final product. In the future, the channels positioning (and the holes) could also be designed in an interconnected fashion, mimicking the fat marbling of a natural muscle tissue. These would no longer be cylindrical individual channels, but a hollow and porous sheet-like channels network.

1.2. Cytotoxicity of VeroGray™ polymeric resin

All the device prototypes were fabricated using Stratasys® proprietary polymeric resin VeroGray™⁸², the only available material in our 3D printing facilities. However, this is not material specifically designed for cell culture, and its full composition was not disclosed by the manufacturer. Therefore, there was no information on its possible cytotoxicity effects.

So, to assess whether VeroGray™ could actually be used in the cell culture experiments of this project, we carried out an extracts-based indirect cytotoxicity assay adapted from ISO 10993-5:2009⁸³. C2C12 is a mouse myoblast cell line⁸⁴ and was chosen for this and other assays, because it represents an adequate cell type for testing cultured meat production, exhibiting fast growing rates. Briefly, the device was immersed in culture medium (DMEM), under dynamic culture at 37 °C for a week, to mimic the real scenario in which it would be tested in subsequent cell culture experiments. The aim of this assay and experimental setup used (Figure 11A) was to evaluate whether the resin would degrade into and/or release any cytotoxic compounds under such conditions. To this end, we obtained conditioned medium after 1 day and 7 days of device immersion, and used these media to feed cultured C2C12 cells, while evaluating their metabolic activity along 7 days, comparing against cells cultured in fresh medium. One week was set as the longest time cells would be cultured with the device without any medium renovation.

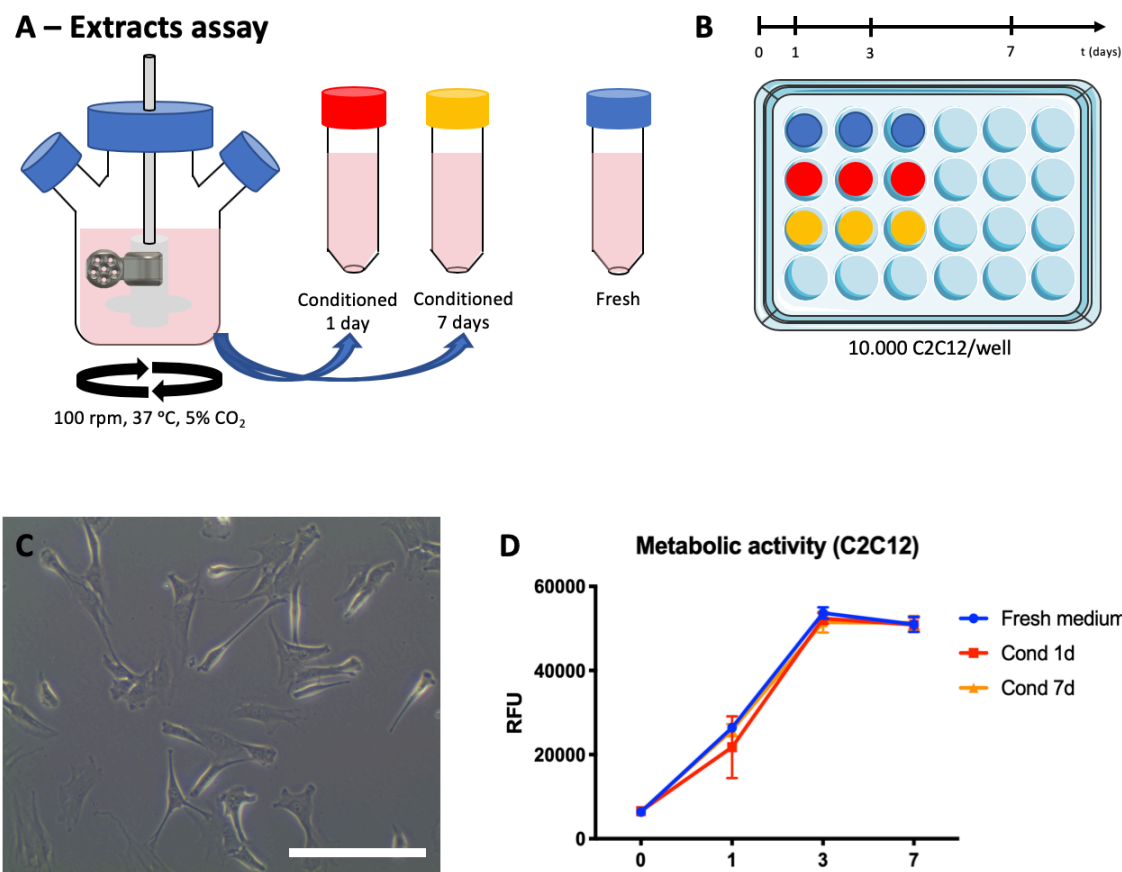


Figure 11 - Cytotoxicity assessment of the polymeric resin (VeroGray™) used for the device production through an extract/conditioned medium assay. A) Setup of the experimental protocol followed to condition medium using DMEM and the device in dynamic culture. B) Setup of the experimental protocol followed to evaluate each medium's impact on the metabolic activity of C2C12 cell line. C) Representative image of C2C12 myoblasts in sub confluent culture. Scale bar: 200 μ m D) Metabolic activity progress of C2C12 cells in fresh medium, conditioned medium for 1 day and conditioned medium for 7 days. Resofurin's presence was measured at day0, day1, day3 and day7 and is represented as Relative Fluorescence Units.

As observed in Figure 11D, C2C12 cells' metabolic activity, along 7 days of culture, was not affected neither by the 1 day nor the 7 days conditioned media (CM). In fact, all three conditions show similar progression with metabolic activity levels increasing around 10-fold between day 0 and day 3. These results indicate that VeroGray™ resin CM does not have any apparent cytotoxic effect on cells, suggesting that it could be used for further cell culture experiments. We stopped evaluating the metabolic activity by day 7, as by that time a plateau was observed in all conditions, suggesting that cells had reached confluency and probably were starting to die due to the lack of nutrients. This assay also confirmed that C2C12 is indeed a fast-growing cell line, as desired for this work.

2. Device validation

2.1. C2C12 myoblasts viability in dynamic 3D culture in the perfusable device

After validating the ability of the device to provide for adequate medium diffusion into the acellular alginate discs, it was necessary to evaluate its performance using cellularized scaffolds. The main purpose of the device is to assure nutrient supply and waste removal in thick scaffolds (>5 mm, at least), supporting cell survival and, ideally, promoting proliferation, ECM production and differentiation, towards formation of an engineered tissue.

Most tissues in the human body rely on an extensive and well-distributed blood vessel network, to get access to nutrients and oxygen, and cells in almost every tissue are located very close to a blood vessel. In fact, the general diffusion limit of oxygen in native tissue is between 100-200 μm ⁷². As most *in vitro* tissues lack a vascularized network, this poses a huge challenge for tissue engineering, particularly when building tissues of clinically/commercially-relevant dimensions. In this cases, cells at the periphery, closer to the nutrients source, will consume them at a faster rate than those in the scaffold core, which will eventually die due to diffusional limitations⁷¹. So, as observed by Lewis *et al.*⁸⁵, an uneven nutrients supply, in *in vitro* tissues, creates proliferation-dominated regions in the outer edge of the scaffold, where cells are closer to the nutrients source.

We have made similar observations in our control bulk discs, left under static conditions. After culturing cells for 3 days under such conditions, we harvested a central slice from the disc, and evaluated cells viability using a live/dead assay. As shown in Figure 12C, there is a 200-1000 μm thick green region at the periphery, where cells remained viable. This region surrounds a necrotic core, with dead cells, which stained red. We believe that the fact that we still have found live cells at thickness higher than the 200 μm limit is related to the low density of a 2 wt% alginate hydrogel. It is reasonable to assume that it facilitates diffusion, as compared to *in vivo* tissues, composed of very dense ECM and several types of cells. Nevertheless, in an attempt to mimic those conditions, we decided to use a very high cell density (14×10^6 cells per mL of alginate, i.e. 14 million cells per disc). For cell culture studies, we had to use RGD-modified alginate to provide a cell-friendly hydrogel. As myoblasts are anchorage-dependent cells, we wanted to reduce the impact of the scaffold on their viability,

by guarantying a 3D microenvironment that would not induce anoikis⁸⁶.

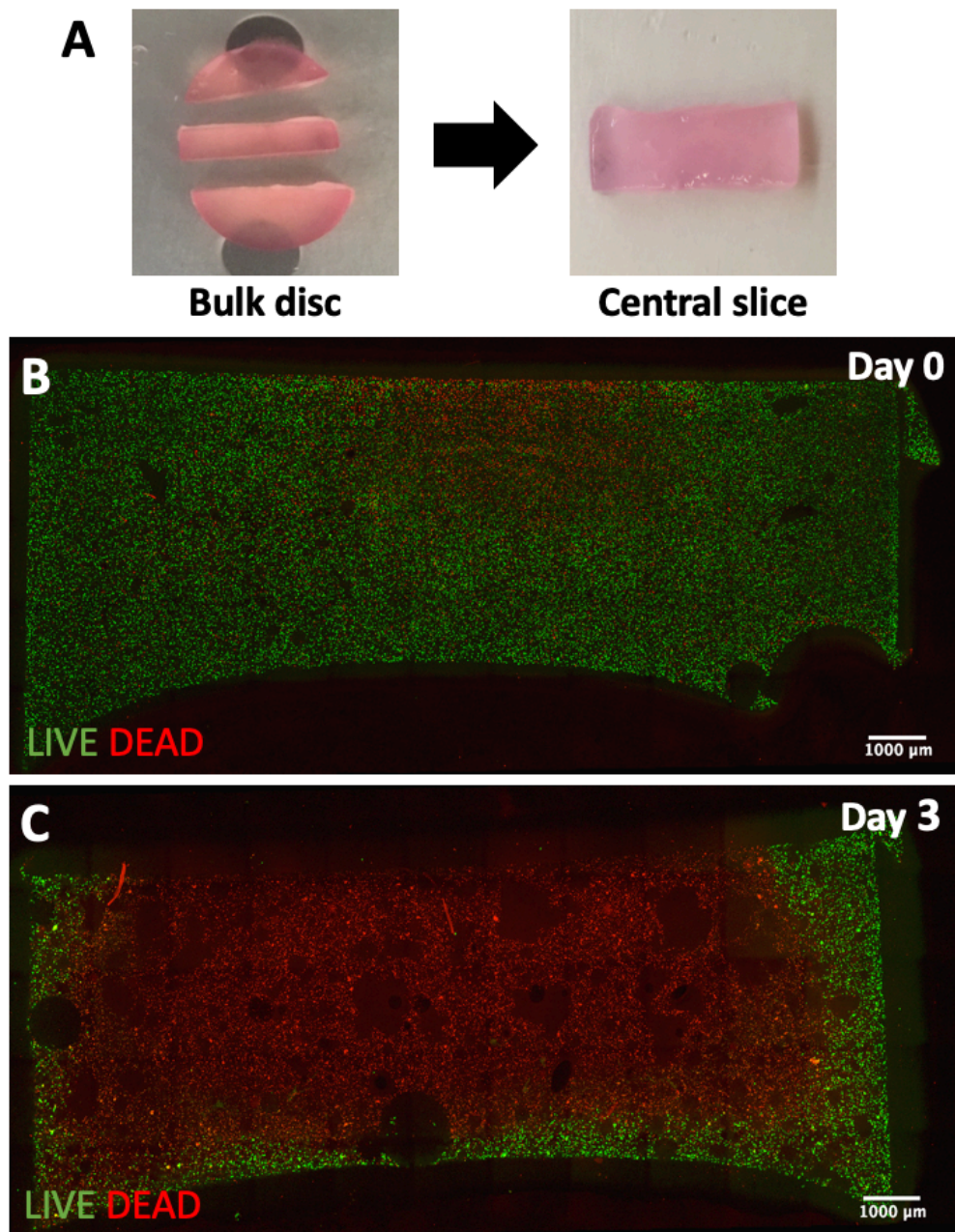


Figure 12 - Bulk disc of alginate 2 wt% cultured for 3 days in static conditions. A) Bulk disc central slicing for live/dead analysis. B) Live/dead image at day0. C) Live/dead image at day3.

Mass transfer is the phenomenon behind nutrients and oxygen supply, as well as in waste removal. This happens because local concentrations are variable⁸⁷ and mass is continuously being exchanged between the culture medium and the cells in the scaffold, where oxygen, nutrients and waste are present in different concentrations. As mentioned before, in static

culture, diffusion dominates. However, in order to improve medium supply, we introduced convection through the rotation of the device in a dynamic culture setup. Convection is a faster mass transfer mechanism than diffusion, as it results from the fluid's movement (the faster the fluid moves, the faster the rate of convection), while diffusion solely relies on concentration gradient as mass transfer driving force⁸⁸. In the presence of convection, mass transport (e.g. of a nutrient) can be described by the convection-diffusion equation⁷¹:

$$J_A = -D_A \times \frac{dC_A}{dx} + v \times C_A$$

where:

J_A is the flow of nutrient A

D_A is the diffusion coefficient of nutrient A in the medium

C_A is the concentration of nutrient A

x is the distance

v is the convective velocity of the medium

Since we are providing the medium a velocity (v), moving it as the spindle rotates at 100 rpm by magnetic stirring, nutrient transport is dominated by convection. In the static culture setup, the convective flow is absent and therefore mass transfer is only dependent on passive diffusion, which, according to Fick's law, is as a function of distance⁸⁸:

$$J_A = -D_A \times \frac{dC_A}{dx}$$

This means that diffusion rate of nutrient A can be promoted by decreasing the distance to be travelled (x). Such fact can explain the big difference observed between bulk and holes-discs controls, where cell viability was greatly improved, which remained both under static (non-convective) conditions (Figure 13C). In the holes-discs, crossed by 6 channels, the distance from the cells at the disc's core to the nutrients source (the media that fills-in those channels) was greatly reduced. This promoted much better mass exchange with the medium, maintaining cell viability during the 3 days even in a static culture, in contrast to bulk discs (Figure 12). In bulk discs, the maximum distance to the nutrient source corresponds to the most central point in the core of the disc. This point is situated at 3 mm from the top or the bottom and at 7.5 mm from one of the lateral sides. However, the fact that the culture is static,

means the disc is laid down with one of its sides inhibited to truly access the medium (top side on Fig.12C).

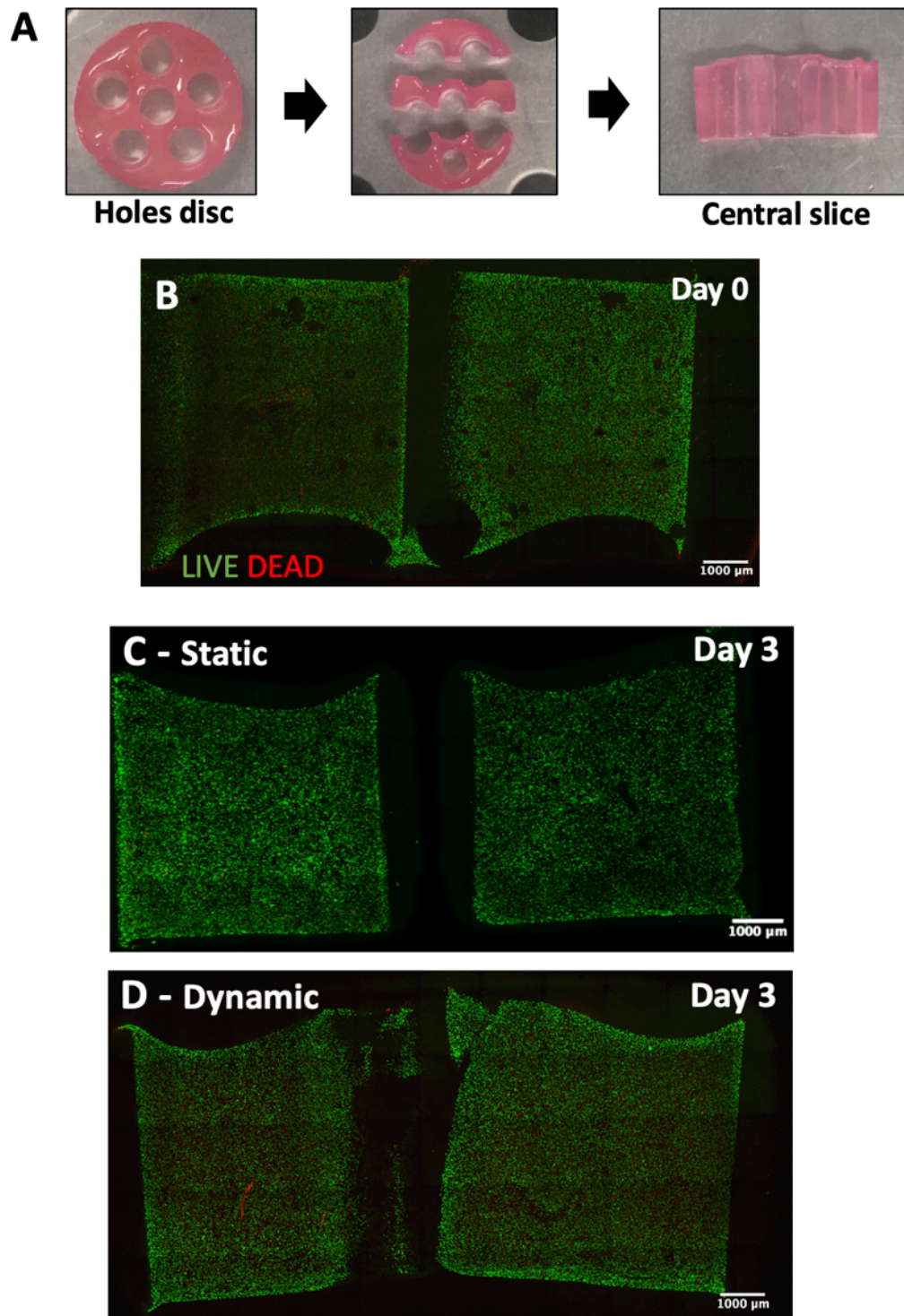
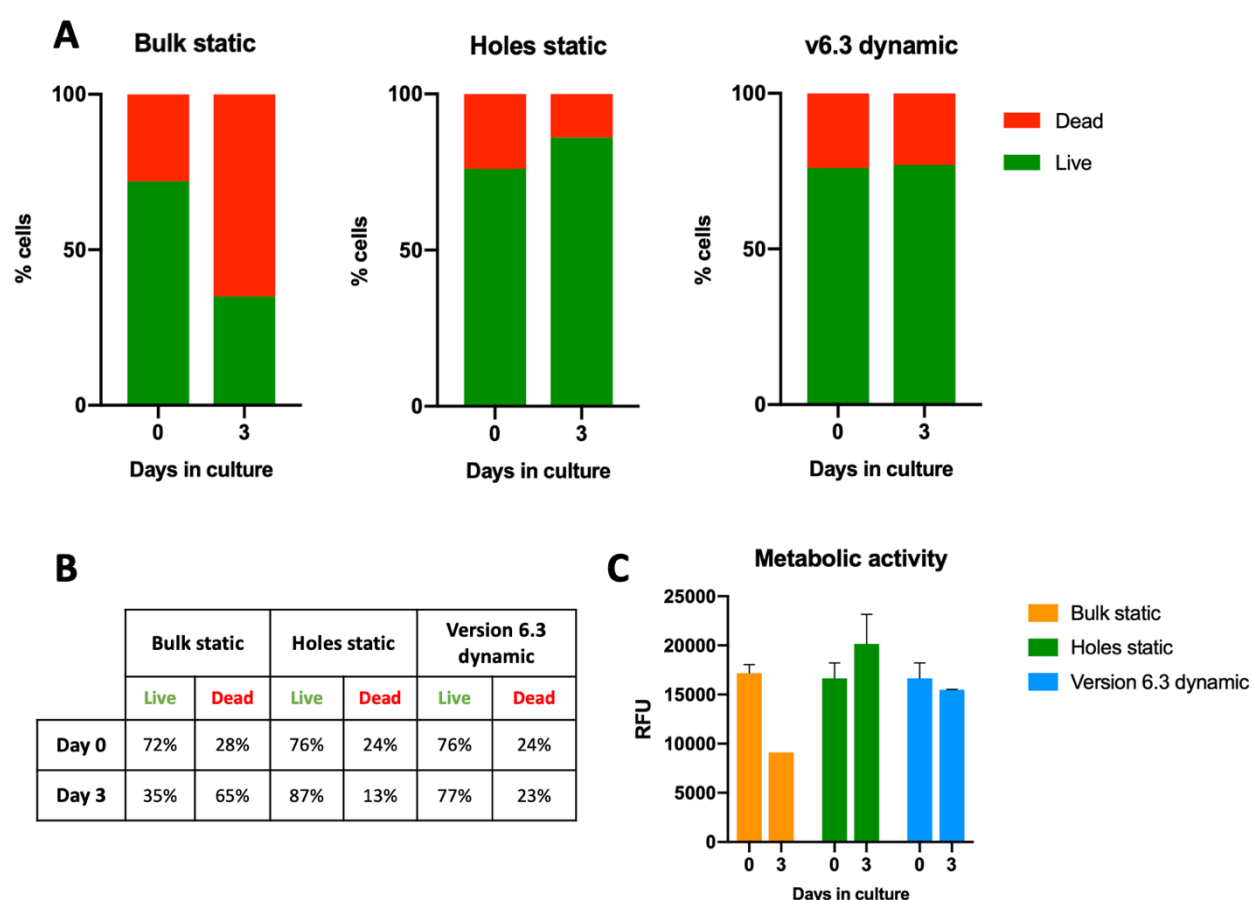


Figure 13 - Holes disc of alginate 2 wt% cultured in static and dynamic condition using version 6.3 of the device. A) Holes disc central slicing for live/dead analysis. B) Live/dead image at day0. C) Live/dead image of static holes disc at day3. D) Live/dead image of dynamic cultured holes disc in device's version 6.3 at day3.

On the other hand, as expectable, the device (version 6.3) was also successful in maintaining cell viability during the 3 days, as observed in Figure 13D and 14A. Cell viability did not change significantly from day0 to day3, with around 76% to 77% of live cells (n=1) nor the metabolic activity (n=3). The device performed much better than the bulk static disc, but slightly worse than the holes static disc, where cell viability (76% to 87%) and metabolic activity both slightly increased along the time. However, these results have to be repeated (more experiments, with more replicates), for a more robust confirmation. Additionally, there were also several technical issues that should be taken into account. First, the discs processed for day 0 analysis were not the same discs processed for day 3 analysis, which might affect the results. Secondly, variability in the cell number embedded per disc must be reduced, from sample-to-sample and between different days, to assure the same amounts of per disc and facilitate comparison. Furthermore, in previous experiments with discs cultured in 6-well plates, we observed some release of cells from the hydrogel to the plate (probably those cells that at the hydrogel surface), which could also affect (lowering) the cell number in discs after of culture, especially in a dynamic setup, where shear stress can exacerbate this.



(Figure's legend in the next page)

Figure 14 – A) Live and dead regions quantifications through the measurement of the mean signal intensity of each channel. Bulk static and v6.3 dynamic (n=1). Holes static (n=2) B) Quantification table with the average values represented in A. C) Metabolic activity assessment of the embedded cells at day0 and day3 of each disc after disintegration of the discs and submitting the cells to the Alamar Blue assay. All discs at day0 have an n=3. Bulk static at day3 (n=1). Holes static at day3 (n=3). Version 6.3 dynamic at day3 (n=2).

Furthermore, we are also not totally convinced with the selected metabolic activity assay. It is challenging to try to assess the metabolic activity of cells in a 3D microenvironment without destroying it to retrieve the cells, because diffusional limitations may occur, masking the results. This would be particularly risky in our samples, due to their large size. Thus, we decided to first disintegrate the discs using EDTA (which chelates Ca^{2+} from the alginate) and trypsin (which dissociates cells) and analyze the suspension of released cells. Clearly, this process can be somehow aggressive, and indirectly affect both cell viability and metabolic activity, so results might no longer effectively represent how cells behave while within the alginate. In fact, as several studies already reported^{89,90}, metabolic profiling of cells in a 3D vs 2D environment changes dramatically. Therefore, the metabolic activity represented in Fig. 14C is not representative of how cells behave in the 3D matrix of the discs. Also, the resazurin assay was performed immediately after the cell suspension being retrieved from the alginate, so most cells were not even able to adhere to the surface. In conclusion, the metabolic activity was mostly assessed on cells in suspension, which is far from being optimal and might provide unreliable results, suggesting that other methodologies should be adapted in future studies.

Overall, the final version of the device confirmed the results obtained in the food coloring experiments and was able to maintain, as desired, the myoblasts viability in a 6 mm thick scaffold during 3 days of dynamic culture. In the future it will be important to evaluate the system's performance for longer culture times. Even though the holes static disc showed a similar performance, we believe, as mentioned before, that the device will present advantages when scaling-up to larger and thicker tissues. Furthermore, as oxygen, often the limiting factor in successful tissue growth *in vitro*, has poor solubility in culture medium (0.2 mmol O_2/l)⁹¹, we believe that the dynamic culture conditions will prove to be more efficient, by promoting continuous re-oxygenation of the medium. Nevertheless, this must obviously be confirmed.

3. Bovine muscle cells isolation and characterization

3.1. Purification of bovine satellite by FACS

The main motivation of this work was to address some of the challenges cultured meat production currently faces. We have used alginate as an edible and animal-free scaffold, but this was cultured with a mouse myoblast cell line, which is non-appropriate for human consumption. In order to produce an edible tissue, we moved into the final stage of the project: the isolation, expansion and characterization of bovine muscle primary cultures, including satellite cells. Satellite cells, as mentioned before, are the muscle stem cells⁵⁵, responsible to produce new muscle tissue by differentiating into myoblasts, which will later fuse into myotubes/myofibers (primordial muscle fibers).

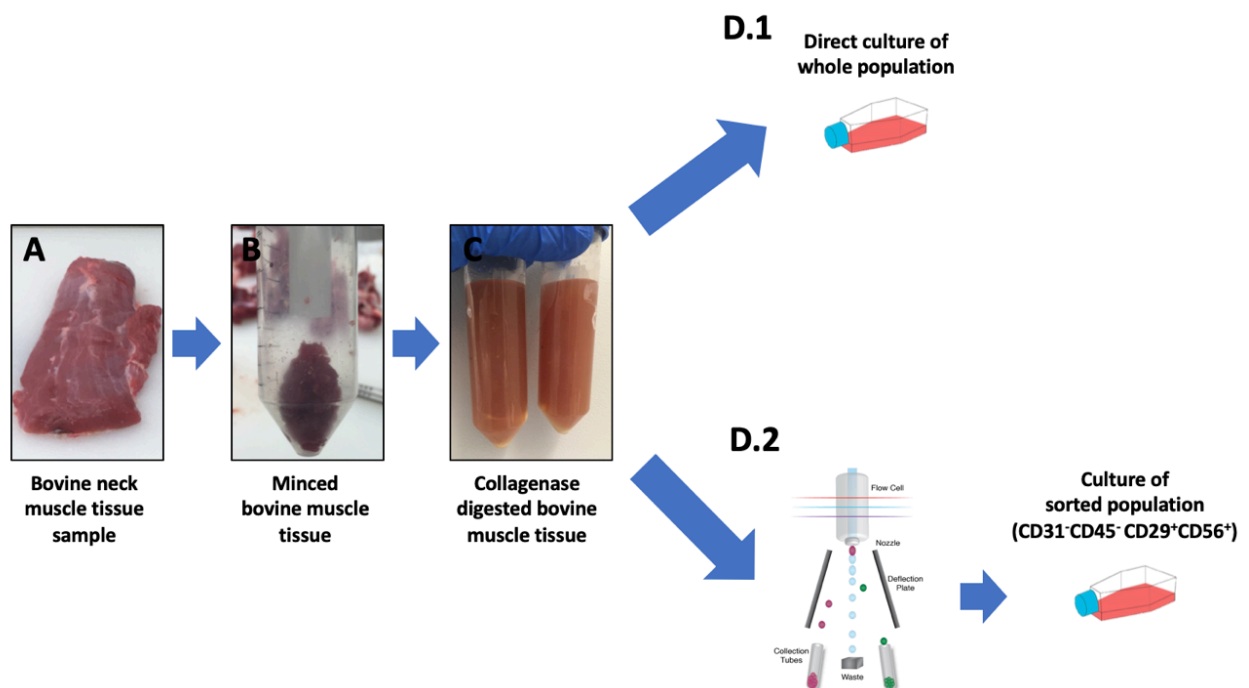


Figure 15 - Bovine muscle cells isolation strategy. A) Bovine neck muscle tissue sample from a 1-year old cow. B) 5 grams of minced bovine muscle tissue. C) Collagenase IA digested bovine muscle tissue. D1) Direct cell culture of the whole population of cells retrieved from the digested muscle tissue. D2) FACS purification of CD29⁺CD56⁺ cell population from the whole population of muscle cells isolated.

To isolate bovine satellite cells from a cow muscle tissue, we have adapted the FACS protocol developed by Ding *et al.*³⁶. It relies on their identification of CD29 and CD56 as positive markers of satellite cells, as they co-stained with PAX7, a transcription factor and the

most specific marker of muscle satellite cells, being crucial for their normal behavior^{95–97}. Endothelial and hematopoietic cells marker CD31 and CD45, respectively ^{92–94}, were used as negative markers. Regarding the isolation procedure, the main adaptations we have made related to the tissue's digestion and RBC lysis. We skipped the double trituration of the tissue that was proposed and introduced, instead, a vigorous pipetting step (30 times) of the muscle sample, to allow maximum release of the cells from the tissue. Additionally, we have also changed the temperature of the RBC lysis step to 37 °C (instead of being on ice), which showed better results in terms of red blood cells removal (not shown).

After isolating the whole muscle cell population from the neck muscle sample (Figure 15), we directly cultured half of the cells onto collagen type I pre-coated flasks or 6-well plates and performed the FACS purification on the other half.

As depicted in Figure 18 A2, fibroblasts were the most predominant cell type on the whole muscle cell culture, which was expected as they account for the majority of native muscle connective tissue⁹⁸. Interestingly, in these un-purified cultures, we have observed spontaneous myotube formation, 21 days after isolation (cells in P4) (Figure 16 A3). This indicates that, although fibroblasts were the most prevalent cell type, myoblasts were also present. Although these are interesting results, we were uncertain if this amount of myoblasts would be sufficient to assure proper muscle fibers formation, in 3D, at a larger scale. Therefore, we decided to evaluate the option of purifying the population, by enriching it in satellite cells, to increase its tissue muscle formation potential.

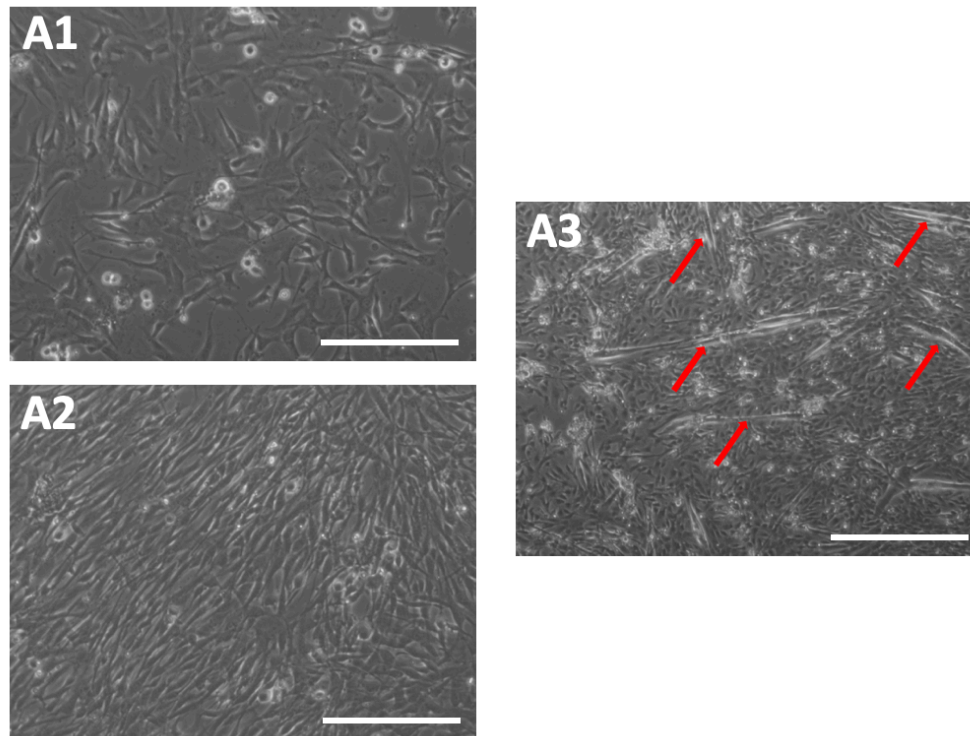


Figure 16 - A1) Whole muscle cell population at P4. A2) Fibroblastic appearance of a majoritarian population at P4. A3) Spontaneous myoblast fusion into myotubes within the whole muscle cell population at P4, at a confluent stage. Scale bars: 200 μm (A1-A2) and 500 μm (A3)

The gating strategy represented in Figures 17A and 17B, and used to increase satellite cells density, trails the following: first, Forward Scatter - Area (FSC-A) and Side Scatter – Area (SSC-A) were used to distinguish cells from tissue debris. Second, FSC – Height (FSC-H) against FSC-A was used to separate single cells from cell duplets, as plotting Height against Area should place single cells in a diagonal linear graphical configuration. Then, CD31 and CD45 were used to further close the gate, by removing positively stained cells for these markers. Finally, plotting CD29 against CD56 allowed purifying the desired satellite population. The unstained cells' analysis, represented in Fig. 17A confirms a proper staining in Fig. 17B, since cells were, as expected, always observed as negative for any marker.

As already anticipated^{99,100}, satellite cells were identified as a very rare cell subpopulation in the whole cell population isolated (~0.1%, see Table D in Figure 17). Satellite cells are called “satellite” because they occupy a peripheral position in the native muscle fibers^{101,102}. While it has been already reported that satellite cells only account for 2-9% of sublaminal nuclei on myofibers for murine adult muscles^{55,103,104}, we did not find any information in the literature regarding satellite cells numbers in bovines.

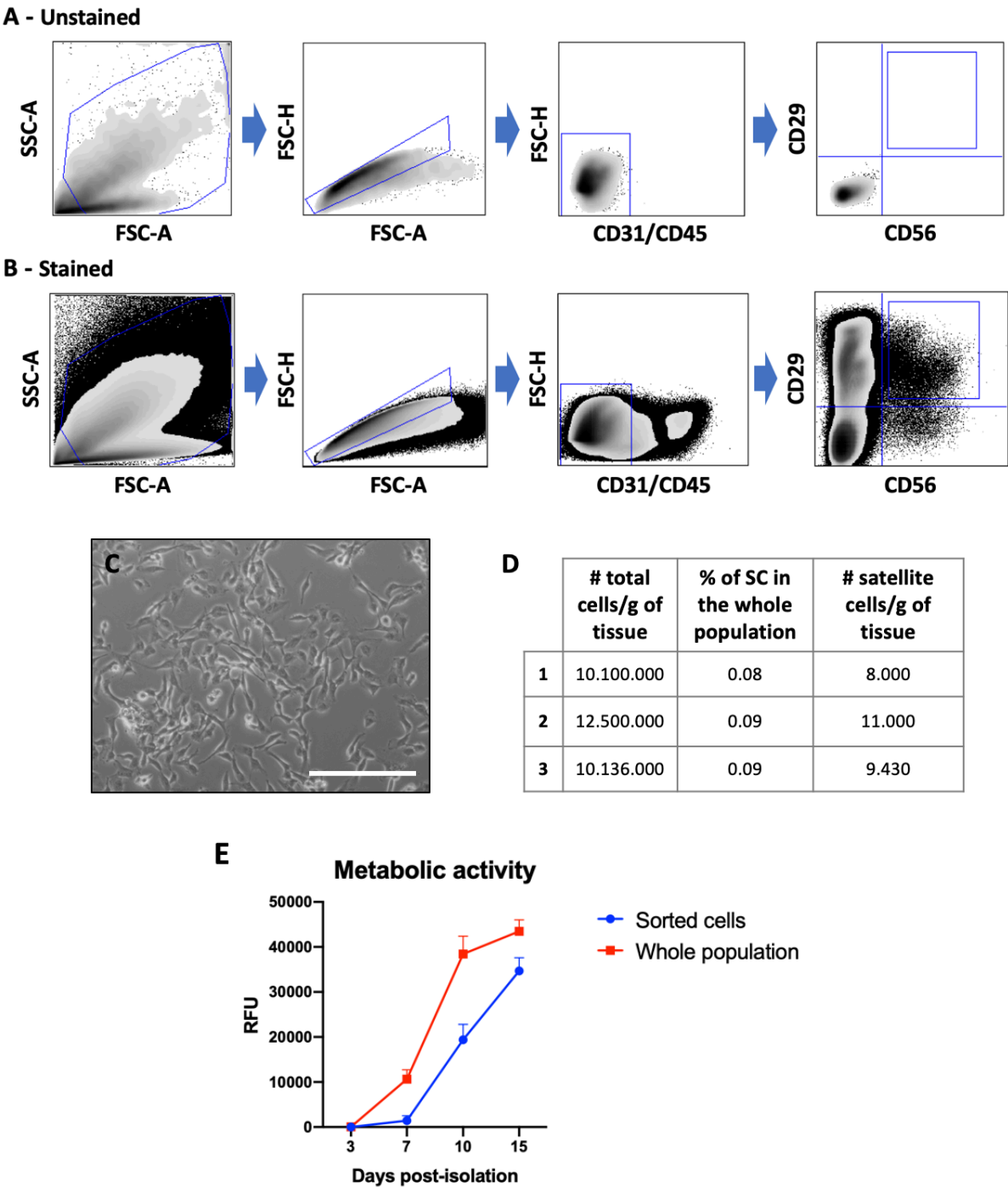


Figure 17 - A) FACS analysis of unstained cells and B) of stained cells from the neck muscle from a 1-year old cow. Gating strategy is showed in each graphic by the blue borders. CD20/CD56 graphs also show the quadrant definition in blue. C) Representative image of sorted $CD31^+CD45^-CD29^+CD56^+$ cells isolated, after FACS purification, in culture at day7 (P0) after isolation. Scale bar: 200 μm D) Isolation and purification yields of 3 different isolation procedures for whole population muscle cells and satellite cells. E) Metabolic activity of whole cell population and sorted cells assessed throughout 15 days after isolation and purification.

Furthermore, in order to estimate cell proliferation of the primary cultures we evaluate their metabolic activity along 15 days, after culturing the cells in collagen type 1 coated 24-well plates. The assay was made in continuum, so medium was changed at each assayed time point. As observed in Figure 17E, although originally seeded at the same cell density (5000 cells/well), the whole population showed a must faster proliferation rate than the sorted CD31⁻CD45⁻CD29⁺CD56⁺ cells. This was not surprising, as the culture was rich in fibroblasts, which are a high proliferative cell type and certainly contributed to the high metabolic activity. Nevertheless, by the end of 15 days, sorted cells achieved similar levels of metabolic activity. Overall, these results suggest successful establishment of primary cultures of bovine cell populations, which was the main goal of this part of the project. However, in future studies, it will be crucial to characterize both populations along the culture in more detail, to evaluate if they maintain the desired phenotype.

3.2. Differentiation of bovine muscle cells populations

Herein, although preliminary, we have made some attempts to better characterize these populations. For cultured meat production, one key feature of the cell source should be the ability to differentiate and fuse into myotubes/myofibers. To compare the myofibers formation potential of both populations, we induced differentiation at 90% confluency by serum starvation¹⁰⁵, and quantified myotubes formation 3 days after. Cells cultured in standard 17.5% FBS supplemented F-10 medium were used as negative controls, in which we did not observe any myoblasts fusion. As represented in Figure 18C, sorted population showed a higher number of myotubes formation, indicating higher potential of myofibers formation, which suggest that it might be worthwhile to pursue with the FACS purification step. However, definitive conclusions regarding the sorted population enrichment in satellite cells cannot be made at this point, since the presented data refers to only one isolation experiment, with only one replicate for each condition. Furthermore, due to time and technical difficulties, we were not able to purify an enough number of cells that would allow us to perform the differentiation experiments sooner (than day 6 post-isolation). We had to wait until sorted cells reached confluency, and did not characterize again those cells by FACS, so we are unsure about their phenotype and stemness state at that time.

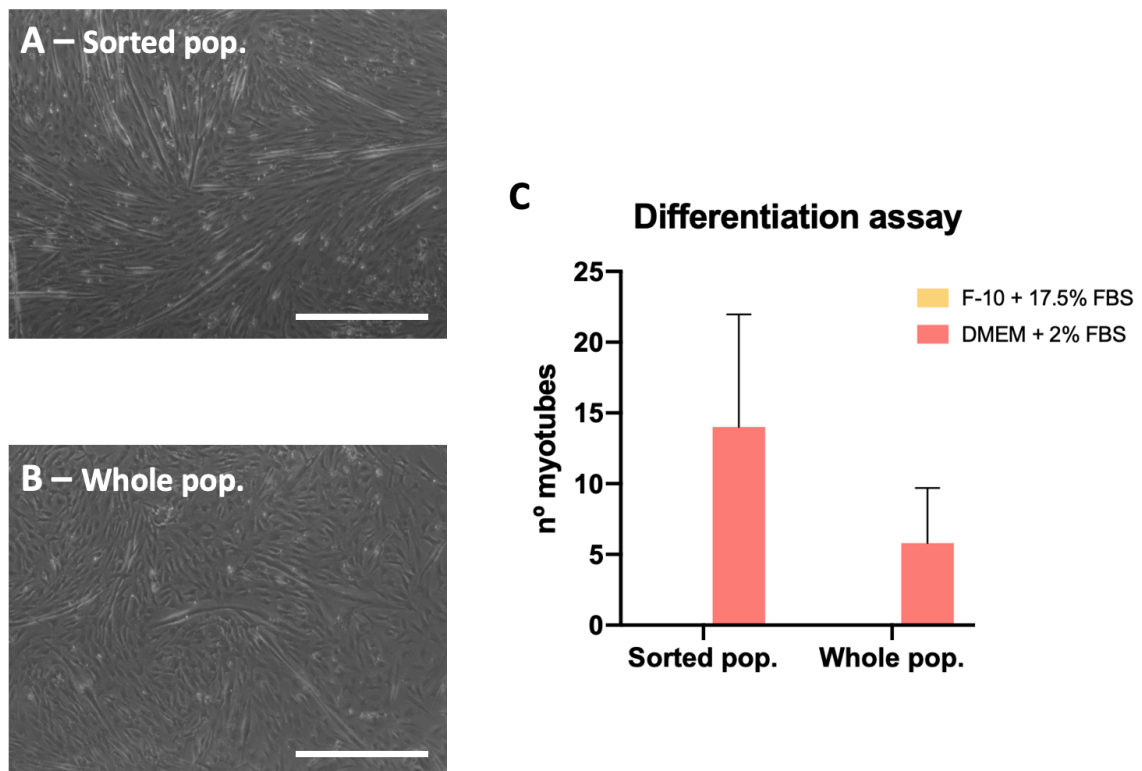


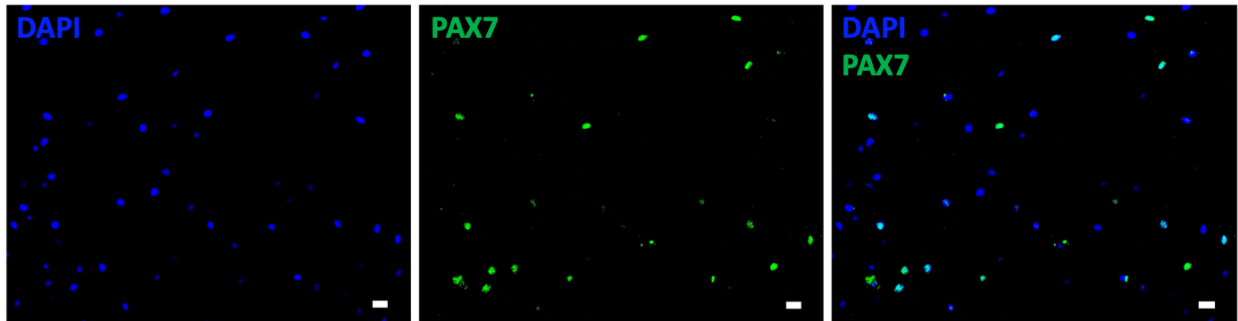
Figure 18 – A) Representative image of sorted cells 3 days after differentiation induction (day 9 after isolation). B) Representative image of whole cell population 3 days after differentiation induction (day 9 after isolation). Scale bars: 500 μ m C) Myotubes quantification of 5 different fields of 1 well for each population. (n=1)

3.3. PAX7 preliminary staining does not show differences between sorted and unsorted cell populations

As mentioned before, PAX7 is the main marker of satellite cells, as it is crucial for the maintenance of their stemness^{95–97} in adult muscles. In an another preliminary effort to further characterize the sorted population of cells, which we believe to be mainly satellite cells, we performed a PAX7 staining following Ding *et al.*³⁶ experimental approach. Surprisingly, we did not achieve the same results. In fact, as depicted in Figure 19B, we did not observe any enrichment in PAX7⁺ cells in the sorted population, having obtained a low level of expression, similar to the one obtained for the whole population culture. Nevertheless, taking into account the lower proliferation rate of these cells and its apparent higher potential to form myotubes, we believe the problem was not related with the FACS purification protocol, but with the staining. Again, because of the low number of purified

cells, we were not able to perform the staining after 4 days of culture, as Ding and his team did, but only a week later. Thus, our results cannot be compared with theirs, and the experiment should be repeated.

A



B

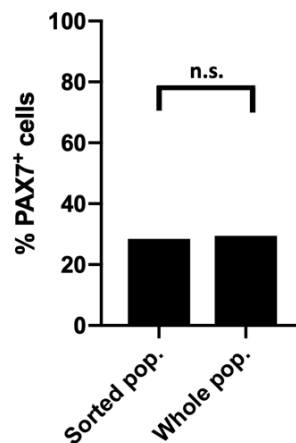


Figure 19 – A) Representative immunofluorescence image for the PAX7 staining in sorted bovine CD31⁻CD45⁻CD29⁺CD56⁺ cells. Scale bars: 25 μ m B) Quantification of the PAX7 staining in sorted cells and in the whole population after 7 days of culture (n=1).

3.4. Differentiation of satellite cells for the production of a beef meatball

As a proof of concept of the cellular agriculture technology ability to produce actual food, we attempted to produce a “beef meatball” using the previously sorted bovine cell population. For this, we have expanded this population up to P4 and induced differentiation (by serum starvation) of 35 x10⁶ cells for 5 days. At the end of this period, most of the cells had already fused into myotubes (see Figure 20A), so we harvested them from the flasks and pelleted down together. For the production of the meatball (see Fig. 20B), the bovine

myofibers were mixed with grated onions, garlic, breadcrumbs, parsley, salt and pepper. Finally, as cells in culture do not contact with blood, the meatball's color was obtained adding to the mixture 15 drops of beetroot juice and half teaspoon of saffron powder. Overall, the bovine myofibers represented 40-50% of the whole meatball's weight.

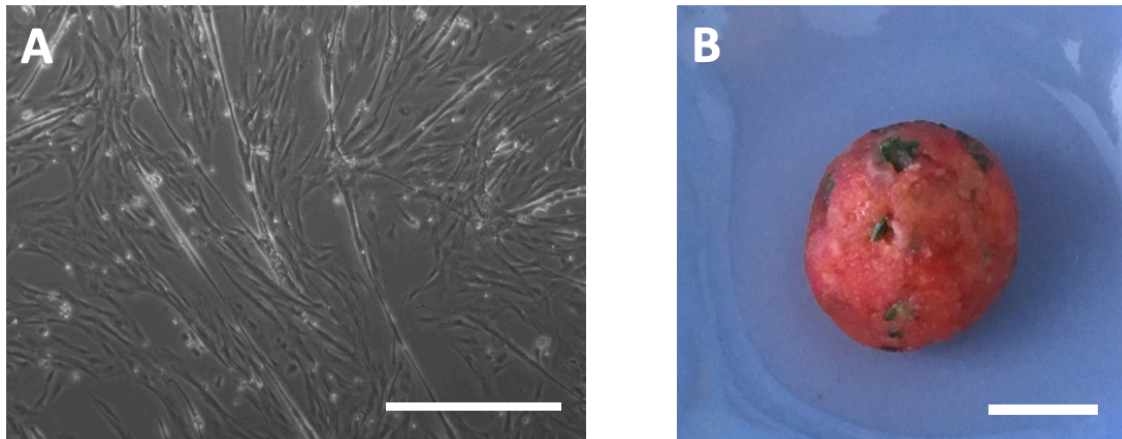


Figure 20 - A) Bovine myofibers formation upon 5 days of differentiation of the sorted cells population. Scale bar: 500 μ m B) Beef meatball produced using the bovine myofibers of Fig. 20A. Scale bar: 0.5 cm

Concluding remarks and future work

Looking at the main goals and main findings of this work, we may conclude that it was a successful project. The final version of the device (6.3) delivered promising results regarding mass transport improvement into the 3D scaffold. This translated into high cell viability after 3 days of culture, which was one major goal. Cells remained alive across the whole 6 mm thickness of the alginate discs, which we did not see in bulk discs. We concluded that the rotation of the spinner-flask was able to efficiently deliver medium through the channels, feeding the cells within the scaffold, which provided a proof-of-concept of the proposed strategy. Nevertheless, there are still several aspects we would like to improve and accomplish in the future.

First, the material used for fabricating the device was not the ideal, namely for future use at larger scale. It showed some problems regarding durability and despite not showing visible signs of cytotoxicity, we worry about its biocompatibility and food gradeability. We believe the material should be further explored and we suggest the use of stainless steel, due to hygienic (washable and sterilizable), durability (re-usable) and technical requirements. This could be achieved by using metal 3D printing, which is a validated technology already available in the market. Moreover, due to time and technical constraints, the number of replicates used for the experiments was clearly insufficient and should be increased to improve the robustness of the study and further validate the device. Also, we recognize the lack of a bulk disc in dynamic conditions as an additional control in order to better sustain the conclusions made regarding static vs dynamic conditions. However, we believe it would not have the exact same dynamic conditions as the holes disc cultured in the device, because this control would be freely rotating in the medium. Furthermore, in order to sustain cell viability during tissue formation, the device will need to hold cells and the scaffold for longer periods of time. This would allow cells to differentiate, organize and produce their own ECM, crucial for the final meat quality and texture. Therefore, we would like to test the device for longer periods of time, for assessing cell behavior and tissue-forming ability in the 3D microenvironment, in more detail. Furthermore, electrical and mechanical stimulation should also be considered as major upgrades to the device in a later stage of development, since this would facilitate muscle formation. Finally, we believe the device would prove its major advantages over the holes static discs when assaying much thicker samples with larger devices. For this, larger

spinner-flasks or a similar type of bioreactor would be needed to be used. Taking into account the main goal of fabricating cultured meat products, this would be a crucial step to take, as it would allow to produce complex meat structures such as steaks, of large enough size.

Regarding the cell source, we have adapted and improved the original isolation protocol, namely by facilitating the release of the cells from the tissue and by improving the removal of red blood cells from the isolated population. Additionally, we have shown that bovine satellite sorted cells have the ability to differentiate into myoblasts and fuse into myofibers, the building blocks of a muscle tissue. However, we are aware there is still much work to be done regarding characterization of the isolated cells. For instance, future studies should include a second round of FACS analysis of cultured sorted cells against CD29 and CD56, in order to further confirm the efficiency of the sorting procedure, and make sure that the obtained cell population is indeed enriched in satellite cells. Assaying the differentiation potential would make more sense after this validation, especially if done soon after isolation and with monitoring of specific markers expression along time. The analysis should also ideally include immunofluorescence staining for Myosin Heavy Chain, the motor protein of muscle thick filaments¹⁰⁶, and the PAX7 staining should be optimized. Nevertheless, in all these assays, the number of experiments and replicates must necessarily be increased. Importantly, we did not perform any transcriptome profiling of the sorted vs. non-sorted cells, and did not thoroughly analyzed cell behavior along time, which would be important assets. For future applications, the ability to establish, and maintain for long periods of time, a bovine myoblasts stable cell line and/or primary cultures of satellite cells would be key to generate enough number of cells for meat production. In the shorter-term, we would also like to have such sources to test our device with a more relevant cell type. This would facilitate attempts to induce myoblasts differentiation in 3D, towards formation of structured muscle tissue. Finally, taking into account the spontaneous myofibers formation observed in the whole muscle cell population, we also believe that it will be important to further study the role of other muscle cell populations, such as fibroblasts, when in co-culture with satellite cells, and analyze their crosstalk.

Another component that should be improved in the future is the scaffolding material, which is challenging if the aim is to achieve thick 3D pieces of meat. The device requires a

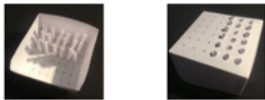
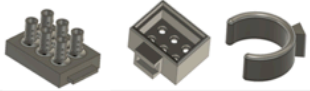
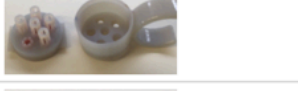
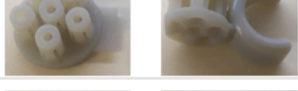
scaffold capable of holding its shape, while retaining cells inside. Herein, we have used alginate because it is edible, animal-free and cheap, and appropriate for high scale production. However, alginate needs to be modified with peptides to promote cell adhesion, and it is not clear whether this modification could be adapted to food-grade standards, and/or would be too expensive and commercially unviable. Therefore, we suggest the exploration of alternative plant-based scaffolds such as spinach leaves, soy sponges and chia seed hydrogels, which might eventually promote proteins adsorption from the media and indirectly allow cell adhesion.

Overall, in this work, a novel perfusable device was successfully developed, which could be modified and scaled-up in the future, for producing more complex cultured meat cuts, thus helping the field of cellular agriculture moving forward. This is extremely important as it would offer people an alternative way to enjoy meat and other animal-protein based foods, without compromising animals' welfare, the sustainability of our planet and the public health.

Annexes

A. Full prototyping process of the device.

Table S1 – Full table of the device's prototyping process. Cl – Channel length; Ecd – Channel external diameter; Icd – Channel internal diameter; Ps – Pore size; Pl – Pore length; Pd – Pore diameter; DbC – Distance between Channels; Rd – Ring diameter

Version	3D Model	Printed Model	Features
0.0		N/A	Paper model. Cubic box with 6x6 hollow, porous and vertical channels.
1.0			Cubic box (13mm x 13mm x 10mm) with 6x6 hollow and vertical channels with squared pores . Back adapter for a peristaltic pump feeding system. Ecd= 2mm; Icd=1mm; Ps= 1mm x 1mm; DbC= 0.6 mm
2.0			Rectangular box (11mm x 16mm x 5mm) with 2x6 hollow and vertical channels with circular pores . Removable ring adapter for a spinner flask central spindle. Ecd= 3mm; Icd=2mm; Pd= 2mm; DbC= 2 mm
3.0			Cylindric box (15mm diameter x 5mm height) with 6 hollow and vertical channels with circular pores . Fixed ring adapter for a spinner flask central spindle. Ecd= 3mm; Icd=2mm; Pd= 2mm; DbC= min. 1.5mm, max. 2.3mm
4.0			Cylindric box (15mm diameter x 6mm height) with 6 hollow and vertical channels with longer pores . Fixed ring adapter for a spinner flask central spindle. Cl=6mm; Ecd=3mm ; Icd=2mm; Pd= 0.7mm; Pl=5mm; DbC= min. 1.5mm, max. 2.3mm
4.1			Cylindric box (15mm diameter x 6mm height) with 6 hollow and vertical channels with longer pores . Fixed ring adapter for a spinner flask central spindle. Cl=7.35mm; Ecd= 3.5mm ; Icd=2mm; Pd= 0.7mm; Pl=5mm; DbC= min. 1mm, max. 1.8mm
5.0			Version 4.0 divided in 3 components with 6 hollow and vertical channels with longer pores. Fixed ring adapter for a spinner flask central spindle. Cl=7.35mm; Ecd= 3mm ; Icd=2mm; Pd= 0.7mm; Pl=5mm; DbC= min. 1mm, max. 1.8mm
5.1			Version 4.1 divided in 3 components with 6 hollow and vertical channels with longer pores. Fixed ring adapter for a spinner flask central spindle. Cl=7.35mm; Ecd= 3.5mm ; Icd=2mm; Pd= 0.7mm; Pl=5mm; DbC= min. 1mm, max. 1.8mm;
6.0			Open version of v5.0 with 6 hollow and vertical channels with longer pores. Double-support ring adapter for a spinner flask central spindle. Cl=7.5mm; Ecd= 3.5mm ; Icd=2mm; Pd= 0.7mm; Pl=4mm ; DbC= min. 1mm, max. 1.8mm; Rd=14mm
6.1			Version 6.0 with 1.25mm thick channels with longer pores. Double-support ring adapter for a spinner flask central spindle. Cl=7.35mm; Ecd= 4.5mm ; Icd=2mm; Pd= 0.7mm; Pl=5mm; DbC= min. 1mm, max. 1.8mm; Rd=15mm
6.2			Version 6.1 with 1mm thick channels with longer pores. Double-support ring adapter for a spinner flask central spindle. Cl=7.35mm; Ecd= 4mm ; Icd=2mm; Pd= 0.7mm; Pl=5mm; DbC= min. 1mm, max. 1.8mm; Rd=15mm
6.3			Version 6.1 with 0.75mm thick channels with longer pores. Double-support ring adapter for a spinner flask central spindle. Cl=7.35mm; Ecd= 3.5mm ; Icd=2mm; Pd= 0.7mm; Pl=5mm; DbC= min. 1mm, max. 1.8mm; Rd=15mm
6.4			4 sites full ring scalable adaptation of version 6.3

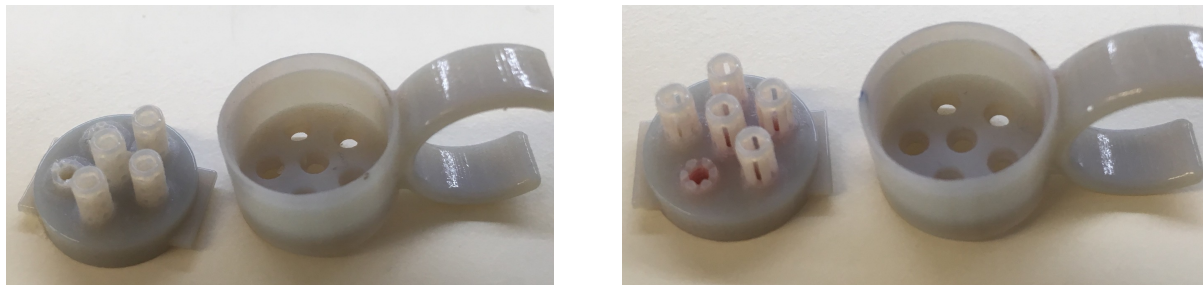


Figure S1 – Left: version 3.0 Right: version 4.0

B. Final device's version (6.3) assembly and setup

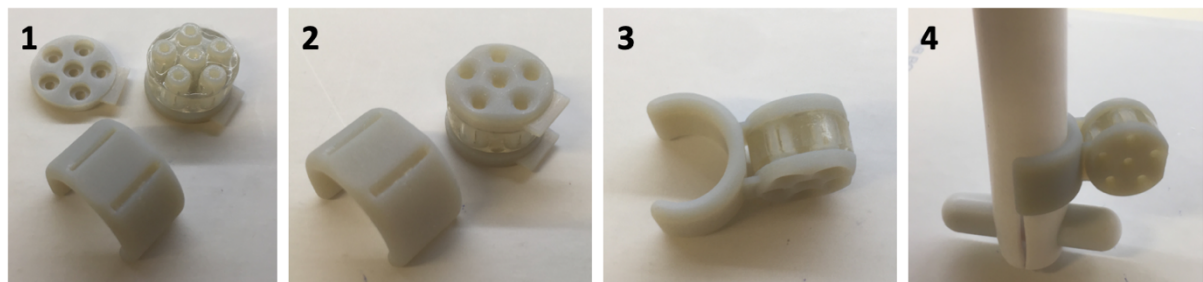


Figure S2 – Assembly of device's version 6.3: 1) Pre-casted alginate disc is introduced in the channels containing component. 2) Lid's incisions fit the channels' tops, closing the device. 3) The two assembled components fit the double-support ring. 4) The whole assembled device is placed in the central spindle of a spinner-flask through the ring adapter.

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