Low-Cost Microfluidic Device to Isolate Pure Populations of Cells

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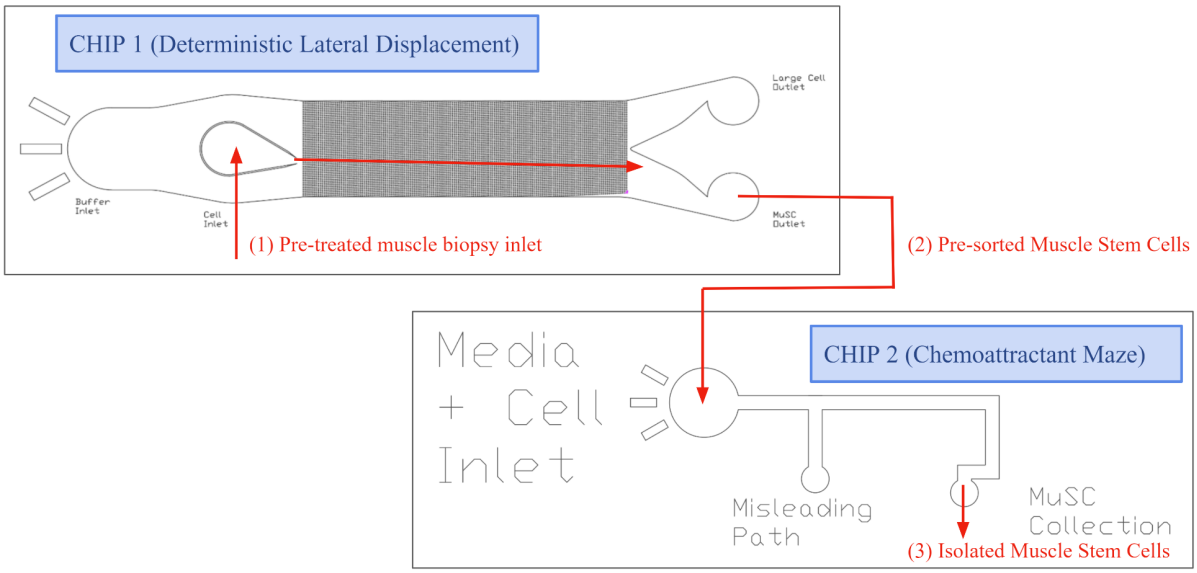

***Abstract*: Cellular agriculture is an emerging field that leverages current scientific methodologies in tissue engineering, animal sciences, and stem cells to create agricultural products and meet the demands of a growing population. Cultivated meat, also known as cell-based or cultured meat, has the potential to address the negative externalities associated with conventional factory farming, including environmental, health, and ethical concerns. One challenge in bringing cultivated meat to the market is the overbearing cost associated with current methods of producing cultivated meat. Here, we address a commonly overlooked costly and time-consuming, but highly necessary, step: cell sorting. Muscle stem cells, adipocytes, and other important cell types must be isolated from muscle biopsies, and the ability to process multiple samples in parallel is a crucial consideration in the process of establishing stable, immortal cell lines. We propose using a combination of microfluidic devices and chemokine gradients as a label-free, low-cost alternative to conventional and costly cell sorting methods, e.g. flow cytometry. To our knowledge, microfluidic devices are not commonly used in current cellular agriculture practices. They not only are extremely affordable and much more accessible to many researchers than conventional technologies, they also offer improved cell viability through non-destructive cell sorting methods, and are highly customizable, depending on the researchers' needs. The proposed microfluidic devices could therefore be an attractive option to accelerate the road from research to retail in cellular agriculture.**

I. INTRODUCTION

The first step in the cultivated meat production process begins with cell sourcing, either through biopsy or incision. To create a stable cell line, a homogenous cell population of the cell type of interest must be obtained from the cell source (Reiss et al. 2021). In other words, the isolation of pure populations of muscle stem cells (MuSCs), adipocytes (key cells for flavoring meat), fibroblasts, and other cell types is central in the cultivated meat process.

Current commonly used cell separation techniques broadly rely on either physical parameters (e.g. size, density) or affinity (e.g. chemical, electrical, or magnetic) (Zhu &

Murthy 2013). Techniques that rely on physical parameters include density gradient centrifugation, field flow fractionation (FFF), and dielectrophoresis (DEP). A major downside of these size and density based methods is the reliance solely on physical attributes. In cell populations with similar sizes and densities, it is extremely difficult to isolate stem cells from non-stem cells. Additionally, these parameters vary from species to species, making it difficult to obtain a certain cell type.

To address these limitations, affinity-based separation methods were developed. These methods utilize antibodies to label surface markers specific to stem cells. These antibodies are then linked to a tag which can be recognized by machines (Zhu & Murthy 2013). Examples of affinity-based separation techniques include Fluorescence-activated cell sorting (FACs), Magnet-activated cell sorting (MACs), and pre-plating. These methods are more commonly used in the cultivated meat industry and cellular agriculture field.

In FACS, antibodies are tagged with fluorescent dyes to cells in mixed suspensions. The cells are then sorted individually based on their fluorescence providing highly pure (95% or higher) cell populations with a throughput of ~30,000 cells/s (Zhu & Murthy 2013; Gossett et al. 2010). An alternative to FACS is MACS, which utilizes antibody-conjugated magnetic beads to bind to specific proteins on cells of interest. The separation is then driven by an external magnetic field. This process allows target cells to be processed simultaneously rather than individually (Zhu & Murthy 2013; Gossett et al. 2010). Lastly, pre-plating separates stem cells from non-stem cells via the extra static adhesion that stem cells exhibit.

However, all three of these methods have key disadvantages, presented in Table 1, suggesting the need for an alternative cell sorting method more suited toward isolating rare stem cell populations.

TABLE I.

Disadvantages of Conventional Cell Sorting Methods (Gossett et al. 2010; Zhu & Murthy 2013)

Methods	**Disadvantages**
FACs	- Expensive equipment and reagents (antibodies) - Requires specialization and training in accordance with the Clinical Laboratory Improvement Amendments (CLIA) - Requires cells to express known biomarkers - Cross-contamination - Slow sorting process - Requires highly skilled or completely dedicated personnel (technical support staff) to use and maintain → increases overhead costs of use
MACs	- Expensive reagents (antibodies) - Requires specialization and training in accordance with the Clinical Laboratory Improvement Amendments (CLIA) - Exerts mechanical pressure on cells and affects their biological activity - Low cell purity - A strong magnetic field is needed (costly) - Slow separation speed - Not sustainable: A disposable separation column is used instead of an ordinary test tube
Pre-plating	- Lengthy culture time - Does not yield a pure population of MuSCs or fibroblasts

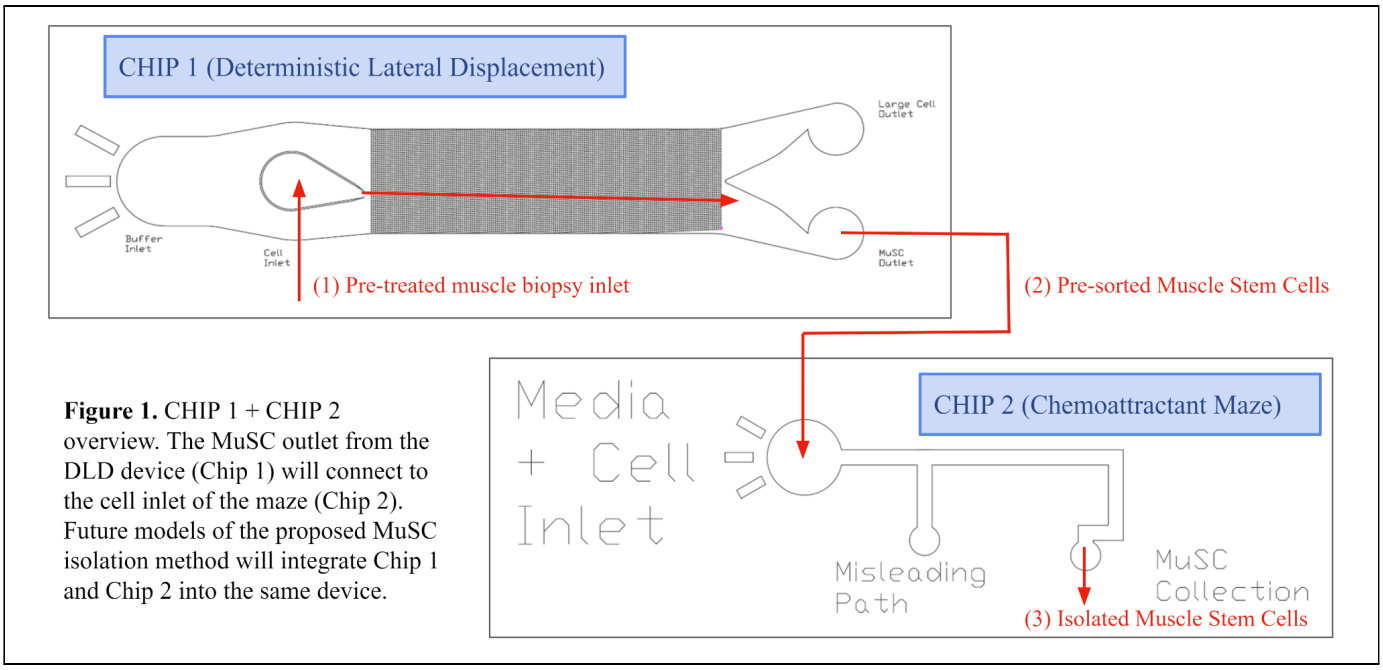

Figure 1. CHIP 1 + CHIP 2 overview. The MuSC outlet from the DLD device (Chip 1) will connect to the cell inlet of the maze (Chip 2). Future models of the proposed MuSC isolation method will integrate Chip 1 and Chip 2 into the same device.

II. MICROFLUIDIC DEVICES ARE AN UNDERUTILIZED RESOURCE

Premise of Sorting Muscle Stem Cells: Muscle stem cells (MuSC) make up only about 2-7% of the total adult muscle cell population, with the percentage decreasing with age (Yin et al. 2013). Mostly made up of their nuclei, MuSCs tend to be much smaller–on the order of 8-10µm–than other cells in the population, e.g. fibroblasts, which may have diameters greater than 15µm (bionumbers.org). The size difference between MuSCs and other cells taken in a muscle biopsy allows for the use of microfluidics and principles of fluid flow for hydrodynamic separation.

Proposed Two-chip Cell Sorting Design: Here, we propose two different microfluidic devices, referred to as CHIP 1 and CHIP 2, that will be connected to each other with Tygon tubing to perform cell sorting with minimal hands-on time (Figure 1). CHIP 1 will pre-sort the MuSCs using a hydrodynamic particle manipulation technique known as Deterministic Lateral Displacement (DLD). Once the majority of other cell types are removed, the sample will be connected to CHIP 2, where a maze filled with a chemoattractant specific to MuSCs will induce chemotaxis of MuSCs toward a designated collection point, completing the isolation of desirable MuSC phenotypes.

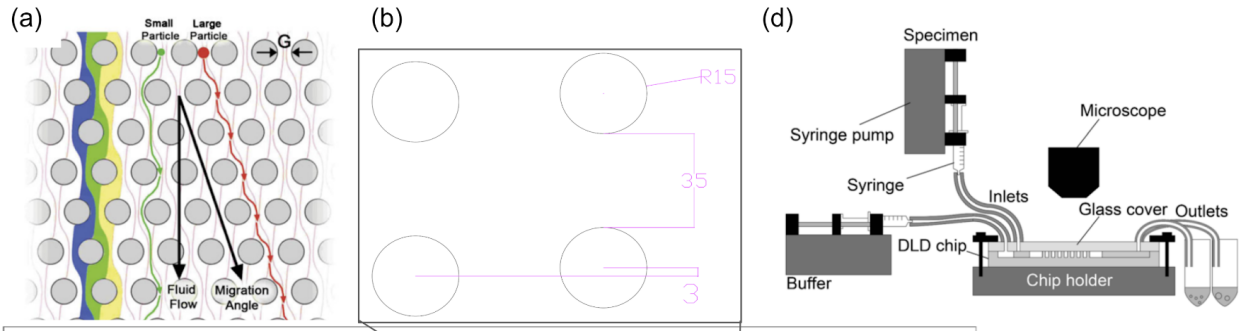

Figure 2. (a) The hydrodynamics of deterministic lateral displacement (DLD) cause large particles to divert from the main fluid streamline at an angle based on the post size, gaps, and offsets (Gossett 2010) **(b)** Post radius 15µm; Post gap 35µm; Post offset 3.135µm. **(c)** DLD device overview. The pre-treated biopsy sample goes into the cell inlet, and is directed at the MuSC outlet. Larger cells will be displaced toward the Large Cell Outlet. The Buffer Inlet keeps the entire channel hydrated. Total device length is 21.50mm. Posts span 11mm of the length. Device height is 25µm. **(d)** A representation of the DLD microfluidic device set-up (Okano et al. 2015).

CHIP #1 - Deterministic Lateral Displacement

Pre-sort: Deterministic lateral displacement (DLD), originally demonstrated by Huang et al. 2004 and repeated many times after (Okano et al. 2015; Zhang et al. 2012; Gossett et al. 2010), occurs when larger particles are pushed laterally from the fluid streamline by a series of incrementally offset posts, while smaller particles are less likely to bump into the posts and flow parallel to streamlines (Fig 2a). Current applications of DLD devices include sorting circulating tumor cells (Okano et al. 2015) and sorting primary cardiomyocytes from neonatal rates (Zhang et al. 2012). The critical diameter, above which cells will be deflected from the main fluid streamline, can be calculated with Equation 1:

$$D_c = 2\eta g\varepsilon \quad \text{(Equation 1)}$$

where η is the parabolic flow constant, g is the post gap, and ε is the shift factor. With a post diameter of 30µm, post gap of 35µm, and 3.135µm shift, the critical diameter D_c is about 9.975µm, about the estimated size of satellite cells (Fig. 2b). D_c was chosen to be below satellite cell size due to cell deformability (cells are not rigid spheres). Figure 2d shows an example of a device set-up.

Expected results and alternatives: While several iterations are required to fine-tune the exact off-sets, post gaps, and post diameters in DLD as well as flow rates, if the CHIP 1

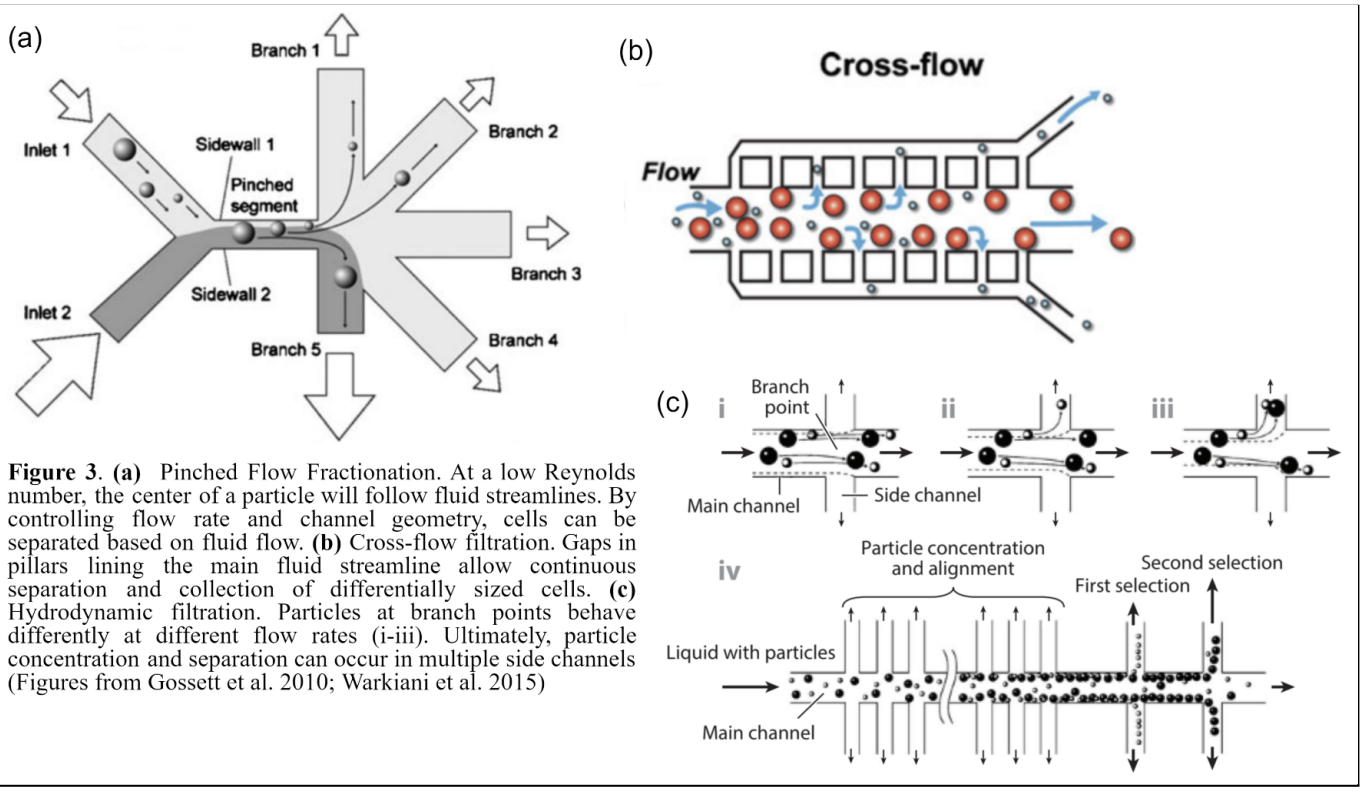

Figure 3. **(a)** Pinched Flow Fractionation. At a low Reynolds number, the center of a particle will follow fluid streamlines. By controlling flow rate and channel geometry, cells can be separated based on fluid flow. **(b)** Cross-flow filtration. Gaps in pillars lining the main fluid streamline allow continuous separation and collection of differentially sized cells. **(c)** Hydrodynamic filtration. Particles at branch points behave differently at different flow rates (i-iii). Ultimately, particle concentration and separation can occur in multiple side channels (Figures from Gossett et al. 2010; Warkiani et al. 2015)

microfluidic DLD device does not remove 50-60% of non-MuSC cell types, then we can use pinched flow fractionation (PFF), crossflow filtration, hydrodynamic filtration or a combination of PFF, crossflow, and DLD (Fig. 3). Fluid flow dynamics can also be simulated on a platform like COMSOL Multiphysics prior to wet lab experimentation.

CHIP #2 - Chemoattractant Maze to Isolate MuSCs: Since DLD microfluidic device is unlikely to isolate a pure population of functional MuSCs, CHIP 1 will be connected to a second chip with a maze that allows MuSCs to form chemokine gradients that initiate chemotaxis toward a collection area. Tweedy et al. 2020 presented evidence that long-range cellular chemotaxis (beyond 1mm) occurs due to sharp, local self-generated gradients produced from degrading chemoattractants present in the media. The migration patterns depend on chemoattractant diffusion rate and flux, rather than quantity.

SDF-1α has been shown to induce migration in CXC chemokine receptor (Cxcr)4-positive myoblasts (Griffin et al. 2010; Brzoska et al. 2012), and can serve as the chemoattractant to isolate MuSCs in a simple microfluidic maze (Fig. 4b). The chemoattractant SDF-1α will

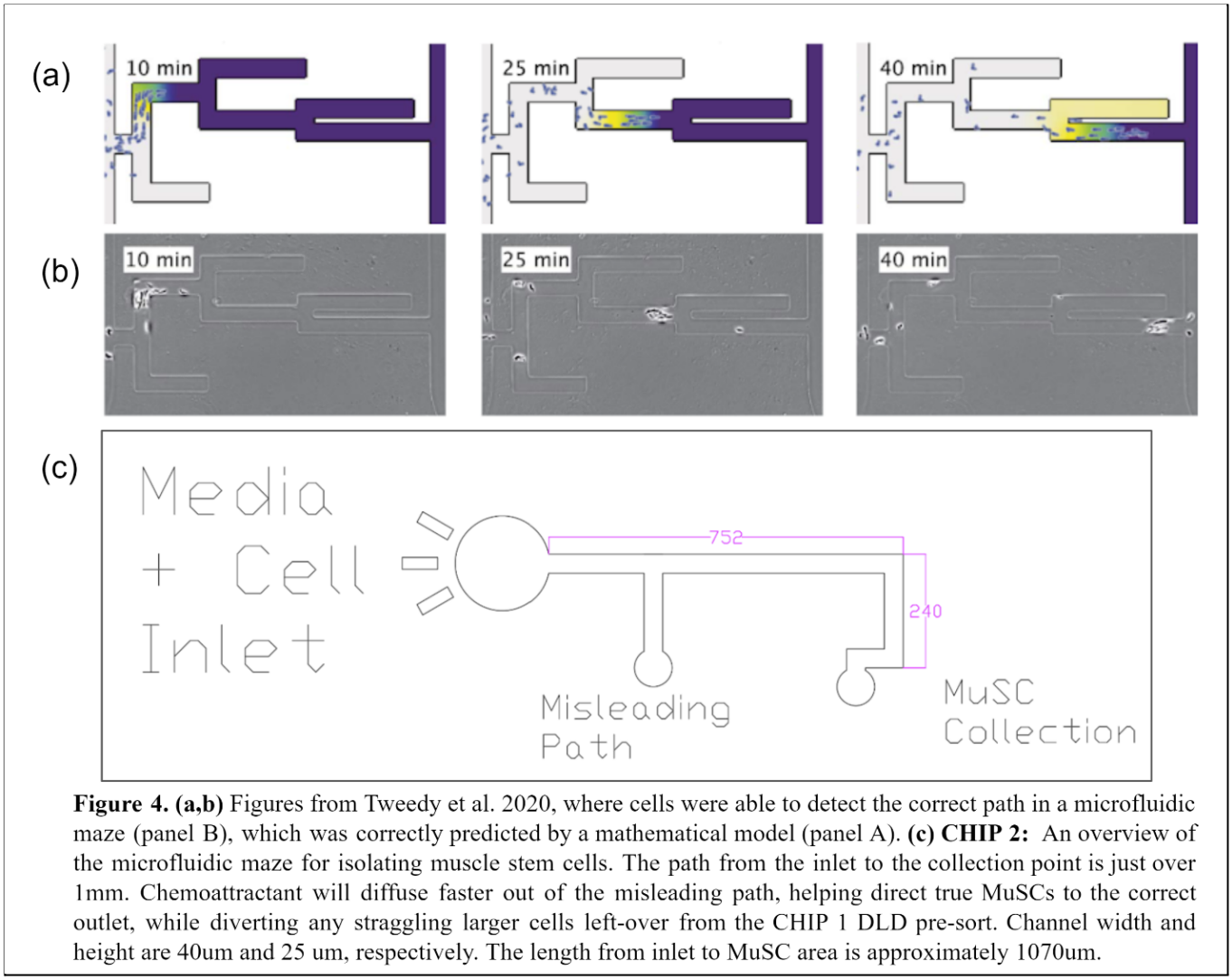

Figure 4. (a,b) Figures from Tweedy et al. 2020, where cells were able to detect the correct path in a microfluidic maze (panel B), which was correctly predicted by a mathematical model (panel A). **(c) CHIP 2:** An overview of the microfluidic maze for isolating muscle stem cells. The path from the inlet to the collection point is just over 1mm. Chemoattractant will diffuse faster out of the misleading path, helping direct true MuSCs to the correct outlet, while diverting any straggling larger cells left-over from the CHIP 1 DLD pre-sort. Channel width and height are 40um and 25 um, respectively. The length from inlet to MuSC area is approximately 1070um.

diffuse out of the short branch faster, making MuSCs less likely to migrate into the shorter "misleading" branch.

Expected results and alternatives: If the proposed chemoattractant maze does not isolate MuSCs to > 85% purity, an additional chemoattractant can be added to draw other cell types away from the MuSC collection area. Tweedy et al. 2020 claimed that *D. Discoideum* cellular migration and decision-making could be predicted using computational models, and a similar mathematical model could be made to predict MuSC migration and optimize maze designs. Chemokine expression in muscle cell migration is still being explored, and other alternative chemoattractants to test are HGF and PDGF (Bischoff 1997).

Accessibility: Microfluidic devices are easily designed using AutoCAD, which can be learned with free YouTube tutorials. Masks are printed

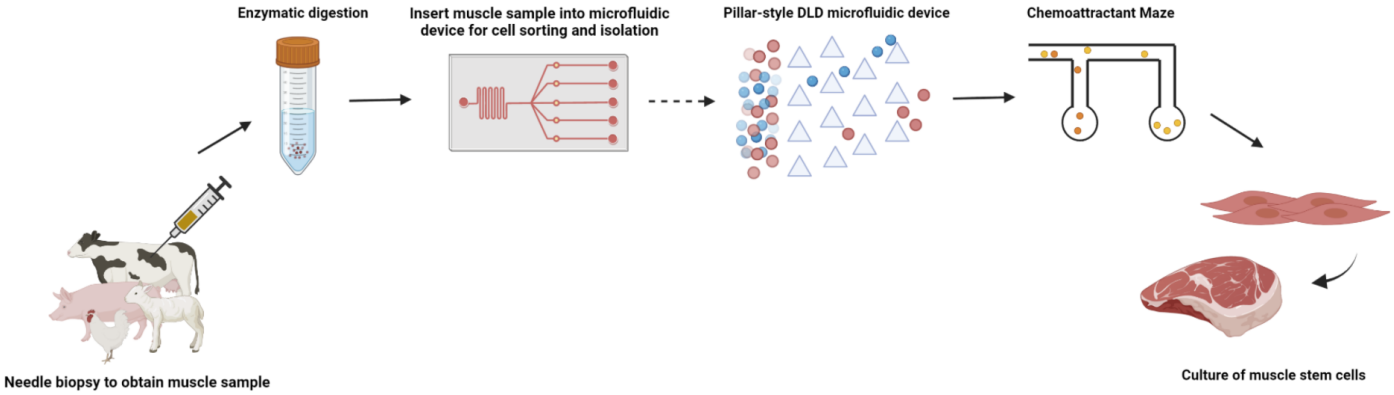

Figure 5. A visual roadmap demonstrating the utilization of the proposed microfluidic resource. The device would sort cells sourced from animal muscle biopsies and isolate MuSCs for downstream cell culture. Figure created in BioRender.

using commercially offered Gerber printing, and wafers are fabricated with standard soft lithography protocols, available in most microfluidic publications (Okano et al. 2015, Zhang et al. 2012). These wafers are reusable for making any number of PDMS devices. Further, Nguyen et al. 2018 presents a method to drastically reduce costs of microfluidic fabrication that could be applied to creating low-cost devices to isolate MuSCs with little technical expertise and hands-on time.

III. POTENTIAL APPLICATIONS

Pre-sorting device before FACS: The fabrication and optimization of CHIP 1 (DLD device) can occur within a few months, but CHIP 2 may require a few more years of research. While CHIP 2 is in development. CHIP 1 can be used as a pre-sorting device to accompany FACS or any other common cell sorting method; CHIP 1 can remove a portion of larger cell types, which will decrease the FACS sorting time, and allow researchers to process more samples.

R&D for immortal cell-based meat cell lines*:* The proposed designs have potential in aiding the development of cell lines for cultivated meat. In early stages of research and development (R&D), many muscle biopsies must be taken from animals of different age, sex, and with different muscle types. Cell sorting is a key step in obtaining the proper cells needed to create stable cell lines. Many CHIP 1&2 devices can be run in parallel, with minimal pre-treatment and without the need for dilution, to isolate desirable cell types that can be cultured readily. Eventually, CHIPS 1&2 can be integrated into the same device, serving as a *single step cell sorting method that requires little technical expertise to run* (Fig. 3).

Beyond Cellular Agriculture: Isolating MuSCs and other cell types is a challenge across cell biology applications. For example, tissue engineers working in regenerative medicine require highly pure populations of MuSCs to combine with therapeutic scaffolds. Therefore, the proposed devices are not solely limited to enhancing the cultivated meat pipeline, but also offer decentralized applications for any researchers in need of a low-cost, easily accessible, non-destructive, customizable method for cell sorting.

IV. EVALUATION OF MICROFLUIDIC DEVICE

Benefits: Microfluidic devices can be used to enrich multiple cell types on the same chip. Secondly, microfluidic devices enable label-free, non-destructive cell sorting and isolation–no antibodies (used in FACS) or microbeads (used in MACS) are required. This method is also less expensive–no bulky equipment, such as FACS machines, is needed, and the device can be operated with little technical expertise (i.e. no trained technicians are necessary). Antibodies and MACS beads are also costly (Table 3). Additionally, the device can save time by operating in parallel; the processing time for a single sample is estimated to be around 20 minutes.

Drawbacks: This method will require many design iterations for each specific application such as isolating just the muscle stem cells, enriching other cell types, adding chemoattractant chips, etc. Moreover, cell deformability is difficult to predict. Lastly, DLD may involve a resulting dilution of the sample as particles are separated into coflows.

Opportunities: Although DLD may not result in a pure population of muscle stem cells, when coupled with another sorting method like FACS, it can reduce the total time of sorting while still allowing for processing of more samples. Importantly, CHIPS 1&2 are extremely accessible to any researchers, especially those involved in molecular and cell biology, and tissue engineering and regenerative medicine. Designs are highly adaptable to the specific research questions being pursued.

V. ROADMAP FOR RESOURCE UTILIZATION

When analyzing the role of the microfluidic device in the cultivated meat production process, as stated in Section III, it can be used as either a pre-sorting device before FACS or eventually as an alternative to conventional stem cell separation technologies. Figure 5 visually demonstrates the step in which our proposed solution will have the greatest impact.

VI. PRELIMINARY TESTING DATA

Due to COVID-19 access restrictions to the cleanroom, the AutoCAD designs and support from literature serves as our preliminary data (See Figures 1, 2, 4).

VII. MEASUREMENT AND EVALUATION OF RESOURCE SUPPLY CHAIN

All materials can be easily sourced from research vendors, including Eden Tech–a high-impact company that specializes in low-energy and low-resource consuming cleantech for microfluidics. Most pieces of the device are reusable, e.g. syringe pumps and fabricated wafer molds, decreasing the costs substantially for running multiple samples in parallel.

While microfluidic devices are typically fabricated using polydimethylsiloxane (PDMS) and SU-8 Photoresist, Nguyen et al. 2018 presents a method to greatly reduce the cost of fabrication through methacrylate (MA), LED-UV light, and Flexdym™–a cleantech Eden Product that has optical transparency and pattern fidelity comparable to that of PDMS. Unlike PDMS, though, Flexdym™ does not require plasma treatment to bond to the glass slide. The low-cost alternatives to microfluidic device fabrication presented by Nguyen et al. 2018 lowers the barrier for entry into the cellular agriculture space from a wider and more diverse community.

TABLE 2. Microfluidic Device Material Costs

Material	**Cost**	**Possible Supplier**
PDMS	~$200/kg	Sigma-Aldrich
Flexdym™	$25/sheet 15cmx15cm	Eden Tech
SU-8 2000 Photoresist	~$400/L	Kayaku Advanced Materials
Methacrylate	$10/15mL	MaterialsWorld
Silicon Wafer	$2 per square inch (200mm diameter)	Sibranch
Syringe pumps	$520 - $1560	New Era Pump Systems

TABLE 3. Cost Comparison of Microfluidic Device, FACS, & MACS (Gossett et al. 2010)

*Includes costs for printing, spinning, irradiating master molds, as well as soft lithography/embossing

Technology	**Overall Cost**	**Labels**
FACS	~$250,000	$300-600 for antibodies
MACS	~$250,000	$600-$1000 for beads (1e8 cells)
Microfluidic Device*	~$6000	None

Table 2 highlights the fabrication costs of a microfluidic device. Table 3 compares the overall cost of manufacturing a microfluidic device to conventional stem cell separation methods, FACS, and MACS.

As shown in Table 3, our solution offers over 40-fold reduction in capital costs to build and purchase the respective cell sorting systems. However, Table 3 does not calculate the full costs for ongoing research, which accumulates due to highly consumable labels such as antibodies or MACS beads. The reusability of the proposed microfluidic designs make it a viable alternative to stem cell isolation with the potential to decrease the price of cell-based meat products, making cultivated meat more accessible to low-resource communities that are most adversely impacted by climate change. After initial capital costs for cleanroom equipment, and fabricating the mold using SU-8 photoresist or Methacrylate, the cost per device decreases to the cost of material for soft lithography or soft embossing: ~$0.88/device with PDMS and ~$0.69/device with Flexdym™.

VIII. SUSTAINABILITY IMPACT

From an ethical perspective, using needle biopsies over incision is more sustainable, since it minimizes cell damage in the animals. Although the sample size from needle biopsies is smaller, the minimal pre-treatment steps, dilution, and reagent-use with CHIPS 1&2 are likely to increase cell viability compared to conventional cell sorting methods. Microfluidic devices may therefore enhance livestock animal welfare, and eventually negate the need for muscle biopsies after establishment of immortal cell lines for cultivated meat.

Furthermore, incorporating microfluidic devices is less costly and more environmentally friendly. Microfluidics deal with very small volumes of fluids–down to femtoliters (fL), a quadrillionth of a liter–requiring less reagent and sample volumes (ElveFlow). While the reusability of the proposed microfluidic PDMS devices has yet to be assessed (but is possible), wafers fabricated to stamp into the PDMS can be reused for years. This is in stark contrast to the more common FACS and MACS, which consume expensive antibodies and beads for each sample.

By eradicating the need for antibodies and other labels, the proposed designs will also reduce animal testing to raise antibodies against relevant targets. Sometimes the desired cell types do not

express well-characterized biomarkers for which there are specific monoclonal antibodies commercially sold.

Using microfluidic devices for cell sorting and isolation in the agriculture industry is overall more animal-friendly, cost-effective, and time-efficient than the current practices (FACS, MACS, pre-plating, etc.).

IX. FUTURE OUTLOOK

Microfluidic devices paired with chemoattractant mazes have the potential to enable rapid, label-free, non-destructive, and low-cost muscle stem cell isolation with high purity. Once CHIP 1 and CHIP 2 are integrated into the same device, other modalities can be added, such as an imaging chamber to monitor MuSC growth, and on-chip culture chambers. Further, initiating partnerships with cleantech companies like Eden Tech can enable rapid scale-up of manufacturing such devices. Taking advantage of the robust field of microfluidics has enormous potential to reduce cost and aid scale-up of cultivated food production through label-free cell sorting that would ultimately aid in the establishment of stable cell lines. Most importantly, microfluidic devices as a customizable replacement for conventional cell sorting techniques can reduce animal testing, as well as the costs of research and production–in cellular agriculture and beyond–encouraging greater community and consumer support due to increased accessibility.

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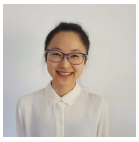
AUTHORS



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