

**Screening Synthetic Hydrogels for iPSC-Derived
Cardiomyocyte Differentiation and Maturation**

By

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This dissertation is dedicated to my parents, my sister, my brother, and my husband.

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Abstract

Stem cells present a unique potential in tissue engineering and regenerative medicine applications, especially since the rise of induced pluripotent stem cells (iPSCs) as an autologous source. Cardiomyocytes can successfully be differentiated from iPSCs, opening up many possible therapeutic avenues for iPSC-derived cardiomyocytes (iPSC-CMs) such as applications in drug screening or disease modeling. However, widespread use of iPSC-CMs for these applications has been limited by the heterogeneity of differentiation efficiencies, the lack of scalability of cell manufacturing, and the limited maturation of iPSC-CMs. Cells are highly susceptible to their microenvironment, including their culture substrate, as physical cues can direct cell fate. Therefore, there is a need for the development of a defined substrate specific to iPSC-CM differentiation and maturation. It is also critical to understand cell responses to substrate properties to elucidate material conditions that give rise to targeted cell behavior. Greater understanding of the dynamic relationship between material composition and cell behavior would allow materials to be rationally designed for specific cell culture outcomes.

In this dissertation, PEG hydrogel formulations were developed and optimized specifically for iPSC-CM differentiation and maturation. First, we evaluate the ability of PEG hydrogels to support iPSC-derived cardiac progenitor cell (iPSC-CPC) adhesion, survival, and differentiation. Cell behavior on PEG hydrogels is directly compared to that on the traditional cell culture substrate Matrigel. PEG hydrogels yield greater iPSC-CM differentiation efficiency along with greater reproducibility across batches and replicates. Next, we investigate the ability of PEG hydrogels to support iPSC-CM maturation by

performing a multi-variate, multi-screen study incorporating optimization modeling. Two distinct but similar PEG hydrogel formulations yield greater iPSC-CM maturation. The use of the optimization model contributes to the rapid identification of promising PEG hydrogel formulations for iPSC-CM maturation. Finally, we study the effect of PEG hydrogel surface topography on iPSC-CM differentiation and maturation. PEG hydrogel surface topography is manipulated by using a soft photolithography approach to transfer a defined pattern. The surface topography directs iPSC-CPC alignment, though this cell alignment is gradually lost as cells aggregate in culture. Short-term cell alignment still has a long-term impact on intracellular and intercellular organization, through myofibril and nuclear alignment. Taken together, the work presented here contributes to the field of tissue engineering, high-throughput screening, and biomaterial discovery, while establishing the groundwork for novel screening approaches and alternative materials for iPSC-CM manufacturing.

Chapter 1 : Screening of Biomaterials for Stem Cell Culture Applications

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1.1 Abstract

Stem cells have a considerable role to play in future biomedical breakthroughs due to their therapeutic potential. As stem cells may be studied in a variety of different applications, a 'one size fits all' approach to the stem cell culture substrate is not appropriate. Different biomaterial formulations may be necessary in different contexts. Screening can help identify biomaterials for specific applications to harness stem cells' full potential. In this review, we cover experimental setups appropriate for screening applications, as well as data collection tools for both material and cell characterization. Finally, we cover high throughput data processing techniques, emphasizing the potential of introducing machine learning (ML) techniques into the analytical process. With increased use of ML-based analytical techniques, biomaterial screening has the potential to contribute to the rapid development of biomaterials for targeted stem cell applications.

1.2 Introduction

Stem cells are increasingly being used for their therapeutic potential, ability to model diseases, drug screening applications, or for expanding developmental understanding [1,2]. However, as stem cells respond to numerous environmental cues simultaneously, a major challenge is developing the appropriate surrounding environment during *in vitro* stem cell culture. This involves manipulating components in solution, including the addition of any soluble cytokines and growth factors [3,4]. It also involves defining the stem cell culture substrate, including material composition, topography, degradation, and mechanical properties [5]. The large number of possible variables associated with the stem cell culture substrate highlights the potential value of applying screening techniques to identify appropriate materials for stem cell culture (Figure 1.1). Although screening techniques have seen widespread use mainly in drug discovery in the past, they have been increasingly applied to material development and identification. The original screening applications in material development and identification for stem cell culture applications mainly focused on screening of large polymer libraries for use as stem cell culture substrates [6-9]. This focus has evolved, mainly due to the integration of computational modeling, to predict appropriate polymer chemistry for stem cell culture [9,10]. The use of screening in material identification has therefore transitioned into multi-variate experimental screens to study the influence of identified parameters on specific cell responses.

Cell-based screening assays have seen growing interest in material discovery [11] since the early 2000s [12-14]. For example, screening approaches have been successful

in navigating large polymer libraries, to identify replacements for natural materials such as ECM proteins [15,16]. With specific synthetic materials showing successful application as stem cell culture substrates [17], the growing understanding that many substrate variables influence cell behavior [5] has led to increasing use of screening as a tool to navigate material variables [11,14]. Some notable material variables include substrate mechanics, biochemistry, degradability, topography, and dimensionality (2D vs 3D) (Figure 1.1) [5,18,19].

Some early screening studies focused on the influence of natural ECM-derived proteins. For example, Flaim et al. developed a microarray platform to study the effects of various combinations of five different ECM proteins on mouse embryonic stem cell differentiation towards early hepatic fate [20]. This study identified fibronectin and collagen I as having a strong positive effect on expression of markers associated with hepatocyte behavior [20]. Similarly, Jung et al studied the combination of various ECM proteins on induced pluripotent stem cell (iPSC) derived cardiomyocyte culture [21]. A screening approach resulted in the identification of an optimized ECM formulation to direct iPSC-derived cardiomyocyte differentiation towards a higher purity population [21].

Another set of screening studies has focused on fully synthetic materials, as they offer substantial control over a larger number of substrate variables than natural matrices. For example, Nguyen et al. screened hundreds of conditions and identified alternative synthetic substrates to replace Matrigel in angiogenesis assays, and in pluripotent stem cell expansion [22]. This study showed greater cell-based assay performance for cells cultured on the optimal synthetic substrates [22]. Similarly, Muckom et al. screened

thousands of culture conditions and identified differential sensitivities to culture parameters over time during stem cell differentiation into cells of the central nervous system [23]. Taken together, these examples and others [11,24,25] suggest that biomaterial screening has the potential to revolutionize stem cell and tissue manufacturing. In this review, we will focus on the key considerations at each stage for a screening experiment to be effective, from experimental setup to data collection and data processing (Figure 1.2). This review will focus specifically on biomaterial discovery for the culture of stem cells and their derivatives.

1.3 High Throughput Experimental Setup

1.3.1 Experimental Platforms

Historically, microarray platforms have been used in screening studies as they support testing of thousands of “spots” simultaneously, and have been useful to investigate combinations of biomaterial substrates [11,26]. Cell-based microarray platforms support multiplexed screening studies of chemical and polymer libraries that modulate biological responses [27]. For example, Ruiz et al studied the influence of spatially patterned surfaces on neural stem cells (NSCs) and demonstrated pattern-dependent axon outgrowth and migration over long-term NSC culture [28]. Additionally, microprinting has shown potential in automating material fabrication by efficiently synthesizing a large number of biomaterial formulations. For example, Ranga et al. developed microarrays with over a thousand unique hydrogel conditions to examine how multiple material variables affect stem cell fate [29]. Le et al. developed a platform based on differential wettability of surfaces to enable efficient printing of nearly one hundred

PEG hydrogels on one glass slide for human mesenchymal stromal cell culture [30]. Another more technologically simple platform that is simple, flexible, and universally available is the standard multi-well plate [31]. For example, Sun et al. identified IKVAV-modified silk fibroin hydrogels to support viability and differentiation of encapsulated neural stem cells by performing cell-based assays in conventional 96-well plates [32]. In a separate approach, Jongpaiboonkit et al. addressed the potential complications of variable hydrogel swelling by developing a hydrogel array platform, and demonstrated an ability to simultaneously evaluate tens of synthetic hydrogels for 3D stem cell culture [33-35]. It is noteworthy that robotics and automation have expanded screening applications, supporting rapid material generation and stem cell culture environments requiring minimal user input [27]. For example, microfluidics-based platforms have supported fully-automated stem cell culture and parallel analysis of the effects of culture conditions on stem cell fate [36,37]. Microfluidic platforms also excel at mimicking *in vivo* fluid flow conditions, and have been adapted to establish higher-throughput stem cell culture systems for biomaterials screening [31,38]. The diversity of these experimental platforms highlights the diversity of screening techniques that can be applied for biomaterial discovery.

1.3.2 Material Fabrication

The broad applicability of the aforementioned experimental screening platforms can be limited due to the biomaterial properties and fabrication methods that are realistically achievable. Fully synthetic hydrogels are chemically tunable and typically highly reproducible [17]. Thus, synthetic hydrogels are generally well-suited for screening

studies when compared to naturally derived matrices, which often present inconsistent compositions and properties [17]. For example, polyethylene glycol (PEG) hydrogels can be easily modified through the use of specific input materials, while naturally derived collagen hydrogels or Matrigel are prone to batch-to-batch variability [29], which may complicate their applicability for screening applications. Furthermore, the choice of material synthesis technique may determine its suitability for screening studies. Many hydrogel networks can be synthesized via photo-crosslinking (e.g. PEG, poly(vinyl alcohol), poly(acrylic acid)), which yields stable and reproducible hydrogels. Other materials that are crosslinked via dynamic mixing of multiple solutions (e.g. alginate, chitosan, hyaluronic acid hydrogels) may result in less homogeneous structure, as well as a smaller range of achievable mechanical stiffnesses which limits their use in screening studies [29,39]. The unique properties of certain materials can also restrain the types of platforms used for screening. For instance, the precursor acrylamide is toxic to cells, and thus polyacrylamide has been suitable for 2D culture [40] but limited for 3D applications like cell encapsulation [29].

1.4 High Throughput Data Collection

1.4.1 Material Characterization

Biomaterial screening involves the development of materials with a large range of possible values for each parameter. Material properties will often each be characterized in multiple ways, with new tools being continuously developed to assess these properties. To assess mechanical properties of biomaterials, atomic force microscopy (AFM), rheology, or compressive and tensile testing are often used [41,42]. AFM offers micro-

scale measurement precision, which provides insight on cellular scale material mechanics [41]. However, AFM generates large data files and has a slow scanning speed due to necessary tip-surface contact and high image quality resulting in long acquisition time [43]. These features limit scalability of AFM for parallel processing of multiple conditions, which is generally preferred in screening studies. Rheology, on the other hand, provides bulk material measurements and can offer rapid shear modulus data acquisition [42,44].

Topographical properties of biomaterials may also be assessed using AFM, thereby streamlining material characterization for two distinct variables – mechanical stiffness and cell-scale topography - using one analysis technique [45]. Scanning electron microscopy can also be used for topographical measurements but typically requires dehydration of the sample, possibly affecting the properties of interest, particularly for hydrogels [46-48]. However, other SEM approaches (e.g. environmental SEM, cryogenic SEM) support analysis under environmental conditions that leave material architecture intact [49]. These SEM techniques offer high resolution imaging from which multiple material properties can be assessed (e.g. topography, porosity, fiber dimensions) but are time intensive to acquire [49]. Optical profilometry, on the other hand, provides rapid topographical data acquisition and bulk material topography information, as the scanning area can be quite large with fields of view on the millimeter scale [50,51].

Biochemical properties such as cell adhesion ligand concentrations or material degradation byproducts, have been assessed using fluorescent labeling or spectroscopy [18,52]. X-ray photoelectron spectroscopy or Raman micro-spectroscopy have been used for characterization of chemical composition, although their long acquisition time can be

limiting when applied to a large sample size [11]. Fluorescent labeling of molecules of interest can offer a qualitative assessment of their presence in or on the material, and quantitative measurements can be acquired with the use of standard curves that define the relationship between fluorescence intensity and molecule density [22,53].

Identifying the appropriate analytical tool for a material property of interest is crucial, but the large variability in biomaterials being generated for screening applications limits the utility of a number of these analytical methods. Multiple factors can be limiting when working with large sample sizes (e.g. sample preparation steps, acquisition time, data processing and analysis). For example, AFM is notoriously limited with respect to screening studies due to the long acquisition time and large data files that complicate scale up to a large number of samples [43]. In response, studies continue to innovate material testing methods to improve applicability in large sample screens. For example, Gandin et al. developed low-cost and high-throughput tools, specifically static macroindentation and micropipette aspiration, to measure hydrogel stiffness [54]. Similarly, Zhang et al. developed a rapid and autonomous high-throughput characterization method to assess hydrogel rheological properties and reinforced the accuracy of their post-processing analysis by integrating a machine learning model [55]. Further innovation in material testing, notably in automation, could have important implications in biomaterial screening.

1.4.2 Cell Characterization

A large number of samples can be a challenge for cell characterization, as it requires efficient sample processing. Although there have been great advances in high-

throughput analysis techniques applicable to 3D culture systems [56-59], most characterization techniques appropriate for screening experiments are designed for a 2D cell culture platform. Characterization of cell behavior and cell-secreted proteins in screening studies is generally reliant on either collecting media or cells, but can be supported in some cases without disturbing stem cell cultures. For example, cell metabolism has been readily assessed using non-endpoint analysis techniques [60]. In these cases, stem cell culture media can be processed to evaluate nutrient use or cell-secreted biological molecules. One common example is a real-time cellular bioenergetics assay that can evaluate cell metabolic activity by quantifying oxygen consumption, glycolysis, and ATP production [61,62]. Additionally, label-free imaging of live cells during culture can provide information about coenzymes involved in cell metabolic behavior [63,64]. In one recent example, Desa et al. successfully applied label-free optical redox imaging to identify metabolic shifts during stem cell differentiation and maturation into cardiomyocytes on PEG hydrogels. The cellular metabolic imaging was used to predict cardiomyocyte differentiation purity in a label free manner [64]. While label-free imaging is perhaps the ideal approach to avoid perturbation of the cells and enable longitudinal analyses, dyes can also be used to track cell location and viability during live cell imaging [65].

For more in-depth cell characterization, cells can be harvested or lysed to evaluate biological molecules of interest. Cells can be lysed to extract RNA for quantitative polymerase chain reaction (qPCR) analysis or for RNA sequencing, a sensitive and accurate analytical tool that requires high quality samples, and which can be labor and

resource intensive. These techniques are generally challenging to apply to stem cell culture studies with a large number of experimental conditions, as they involve complex handling and are difficult to multiplex to evaluate hundreds or thousands of samples. The enzyme-linked immunosorbent assay (ELISA) can be used to evaluate protein expression and, although traditional ELISA has limited throughput, microfluidic applications of the assay have extended their applicability to screening studies [66].

Cells can also be harvested and assessed using flow cytometry to evaluate molecules labeled via immunohistochemistry. Although flow cytometry yields reliable quantitative data, the method requires gentle sample care during cell detachment so as to not damage cell samples, and can be labor intensive during sample preparation [67]. Endpoint imaging through immunocytochemistry (ICC) using confocal microscopy can provide supporting qualitative information, though the user is limited in the number of markers that can be observed simultaneously from a single sample. More specifically, use of multiple fluorophores is possible, but can lead to overlap in the emission spectra of the respective fluorophores, thus complicating the imaging results. High quality image acquisition is also a lengthy process which can be limiting when scaling up the sample size for screening purposes [68]. Image quality is also an additional limitation for certain types of biomaterials, due to background autofluorescence [68] or light diffraction in “thicker” materials [68]. The emergence of artificial intelligence (AI) and machine learning (ML) algorithms for image processing provides a series of novel tools that may result in a substantial increase in the utility of immunocytochemistry in stem cell screening applications [69-71].

There is also growing development of tools for label free image or video analysis to rapidly collect information on cell behavior while limiting any effects on stem cell culture. Further development of such tools may allow for greater amounts of data to be collected from one experiment, by increasing the number of timepoints that can be studied. These tools can be provided through open-source software (e.g. ImageJ, CellProfiler) or through commercial software packages (e.g. NIS-Elements, LAS X). Two specific examples of image analysis tools are the OrientationJ plugin, which measures cell orientation using either brightfield or fluorescence images [72], and the MuscleMotion plugin which provides measurements associated with cell contractility [73]. Similar to the aforementioned imaging approaches, these algorithms generate large amounts of data that may require creative AI and ML approaches to become realistically applicable to enhanced throughput stem cell screening.

1.5 High Throughput Data Processing

The study of biomaterials in a stem cell culture context generally focuses either on gaining basic insights into the influence of a material parameter on cell behavior, or on optimizing a material parameter with respect to a desired cell behavior as the outcome. A screening approach is then applied to study multiple variables simultaneously, resulting in factorial experiments and large sample sizes. A growing number of studies are now also incorporating AI and ML tools to develop efficient and powerful screens (Figure 1.3). AI and ML tools have historically been applied in the context of drug screening [74-76] and materials science [77,78] and are increasingly being applied in cancer treatment, especially image processing of cancer cells for detection, diagnosis, and prognosis [79-

84]. Efforts made in those areas could be judiciously translated to biomaterial screening for stem cell culture, as much of the groundwork for data processing and analysis has been established and is continually evolving.

The brief history of modeling tools has generated a number of useful approaches that either have been, or could be, productively applied to stem cell screening. Modeling tools using dimensionality reduction were initially incorporated into screening studies in the early 2000s [85]. Principal component analysis (PCA) and Uniform manifold projection and approximation (UMAP) have been used to reduce the dimensionality of large datasets into smaller datasets while retaining a majority of the information from the original dataset. These dimensionality reducers (PCA, UMAP) can serve as an initial analytical tool, which can then be combined with more complex classifier algorithms (e.g. support vector machine (SVM), k nearest neighbor (KNN), random forest). Classifiers serve primarily to identify relationships within large datasets, and are often applied in the context of large datasets to support sorting of cell populations or predicting cell behavior [86-89]. For example, Vega et al. evaluated an initial 43 stem cell features in response to substrate parameters and used PCA to reduce the dimensionality from 43 to just 3 dimensions, and then applied SVM to classify stem cell populations [86]. Vega et al. used their approach to develop a non-invasive stem cell culture analysis process able to identify pluripotent and differentiating stem cell populations [86]. In another example, an SVM classifier was used to detect and predict mesenchymal stromal cell (MSC) differentiation patterns on substrates of varying geometries [87].

Modeling tools have also played an increasingly important role in predictions regarding cell fate. Design of Experiments (DOE) can be applied to reduce sample size considerably, while still identifying main effects of input variables on output measurements as well as the way in which variables interact [53,90]. For example, Jung et al. identified an optimized PEG hydrogel formulation for cardiomyocyte differentiation using DOE to limit the number of samples screened experimentally [21]. In some cases, a DOE approach can be applied in the initial experimental screen to reduce sample size and then followed by ML models that predict the next sample set by learning from data collected in the initial screen [90]. The user can then define a desired target or threshold to identify “hits”, which are generally the top 5-10% of output measurements with respect to the targeted screening outcome [91,92]. In this way, ML-based techniques can potentially be a powerful and broadly applicable tool for iterative screening to optimize biomaterials for stem cell culture [93]. In another example, a convolutional neural network (CNN) model was successfully adapted to identify MSC lines with high multilineage differentiation potential using live cell imaging results [94]. Similarly, Fan et al. applied a CNN classifier to detect and predict stem cell colony formation during reprogramming [95]. Further, Wang et al. successfully applied a deep learning approach to sort through hematopoietic stem cell populations [96]. ML-based models not only serve as classifier algorithms but are also applied as optimization tools [93]. For example, Yang et al. developed a supervised learning model to optimize stem cell culture conditions during cardiomyocyte differentiation using live cell imaging, focusing specifically on soluble factors as input parameters [97]. Similarly, Zhu et al. optimized neural stem cell

differentiation conditions by applying a deep learning model to live cell imaging data [98]. Taken together, ML models have emerged as efficient optimization tools for stem cell culture and differentiation. Although the focus of ML models has been limited mainly to the effect of soluble factors on stem cell behavior, the techniques could be generalized to other parameters in the stem cell microenvironment, including biomaterial variables. These ML tools could serve to optimize differentiation, and provide a better understanding of a complex process influenced by an immense number of variables, which could not be realistically studied without the aid of ML.

1.6 Conclusion

The appropriate experimental platform, data collection approach, and analytical tools are crucial for the success of a biomaterial screening study. Innovative approaches and novel tools have been continuously developed for each step of a screening approach, and advances in ML-derived tools have emerged rapidly over the past decade. In particular, ML-based modeling tools have been extensively applied in the context of cancer cells [99], drug screening [100], or materials science [101]. Progress made to date in experimental screening, analytical methods, and emerging modeling approaches could result in substantial progress toward biomaterial optimization for stem cell culture [102]. While screening is increasingly used in the context of stem cell culture, it will be crucial to more consistently integrate biomaterials and the physical variables of the stem cell microenvironment for a full optimization of stem cell culture conditions.

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1.8 References

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1.9 Figures

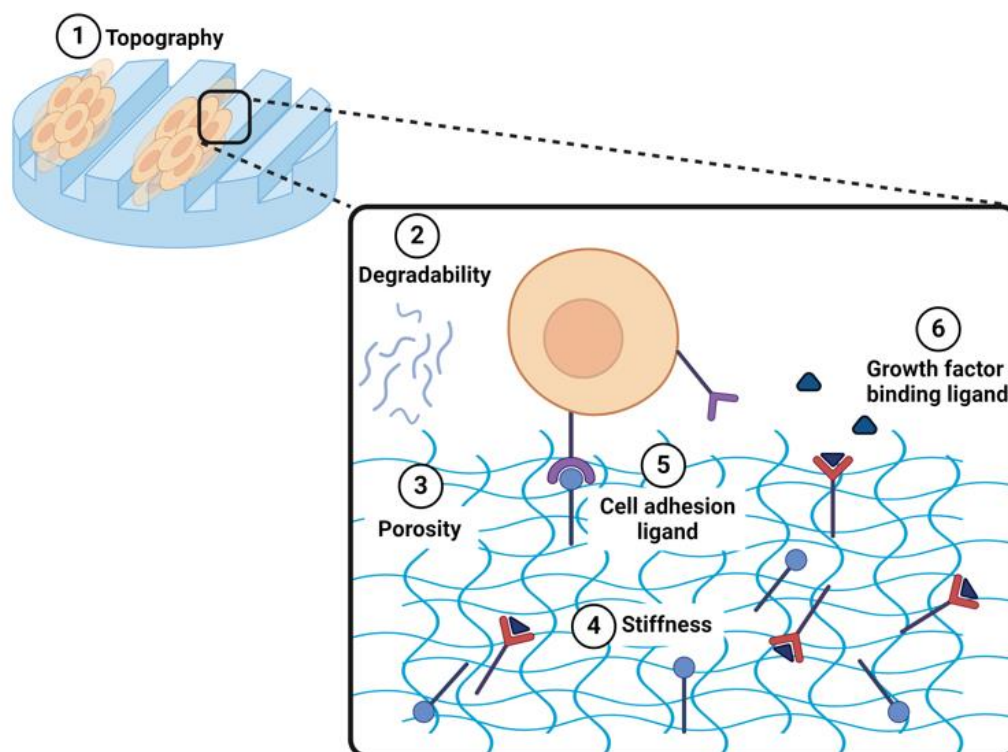


Figure 1.1. Material variables directing cell fate

Synthetic materials offer tunable properties that can be adapted to fit a specific cell type or target cell behavior. Some material variables that are readily modifiable include topography, degradability, porosity, stiffness, cell adhesion ligands, and growth factor-binding ligands.

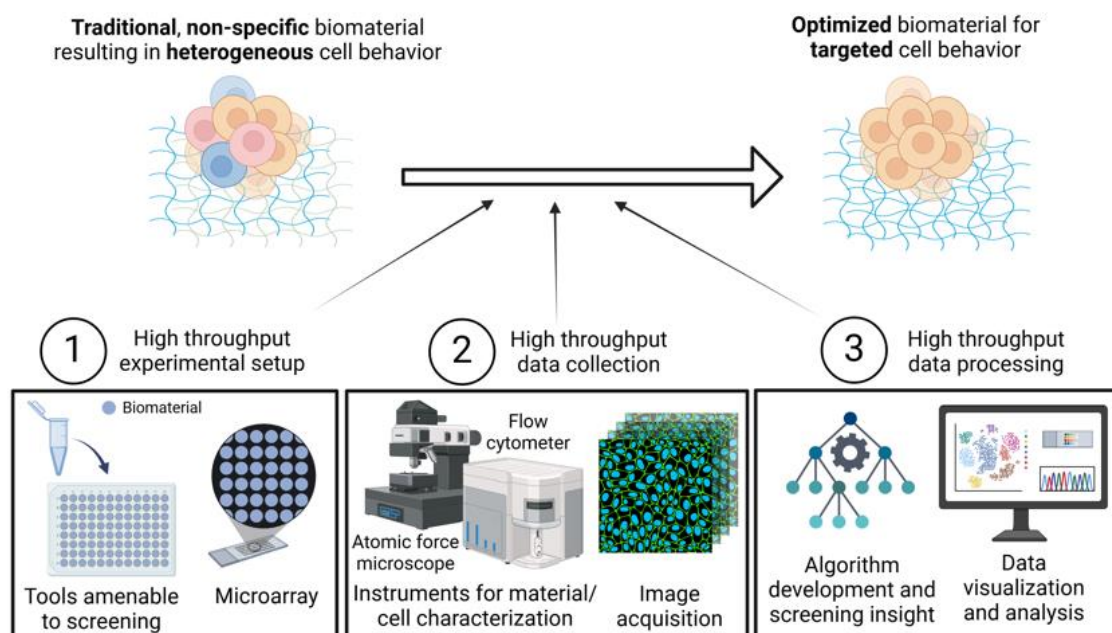


Figure 1.2. High throughput screening workflow for biomaterial discovery

High throughput screening (HTS) can identify optimized biomaterials for specific, targeted cell behavior. HTS can be an especially efficient tool with the correct experimental setup, data collection tools, and data processing techniques.

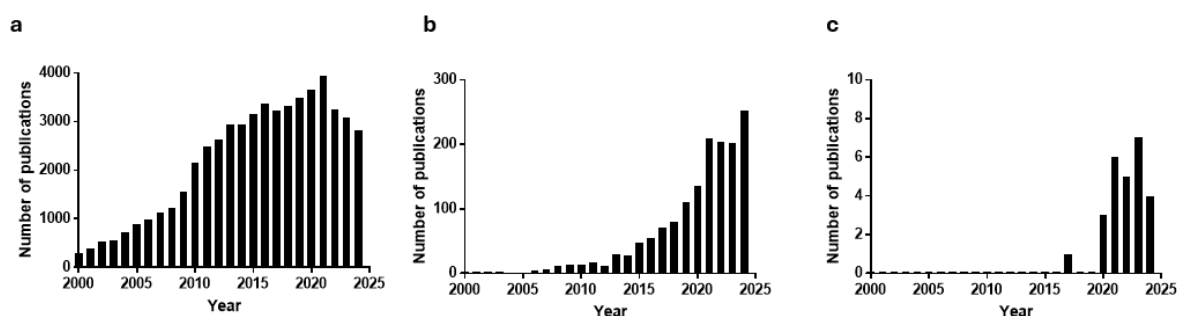


Figure 1.3. Publication trends since the 2000s

Publication trends show an increased number of publications related to **(a)** high throughput screening, **(b)** high throughput screening and machine learning, and **(c)** high throughput screening and machine learning and biomaterials. (Source: Pubmed, 2024)

Chapter 2 : Synthetic Hydrogels Support Robust and Reproducible Cardiomyocyte Differentiation

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2.1 Abstract

Cardiomyocyte manufacturing from human pluripotent stem cells is limited by the variability of differentiation efficiencies, partly attributed to the widespread use of the tumor-derived substrate Matrigel. Here, we describe a screening approach to identify fully-defined synthetic PEG hydrogels that support iPSC-derived cardiac progenitor cell (iPSC-CPC) adhesion, survival, and differentiation into iPSC-derived cardiomyocytes (iPSC-CMs). Our PEG hydrogels supported superior iPSC-CM differentiation efficiency, with a 24 % increase in cTnT expression, and greater reproducibility when compared to cells cultured on Matrigel. By combining our 5-level, 3-variable full factorial screening approach with multi-variate analysis, we showed that all substrate variables manipulated here (adhesion ligand type/concentration, stiffness) had a significant influence on iPSC-CPC confluency and that iPSC-CM differentiation was significantly influenced by adhesion ligands. These results highlight the benefit of synthetic, tunable cell culture substrates and multi-variate screening studies to identify substrate formulations for a targeted cell behavior.

2.2 Introduction

Human induced pluripotent stem cells (iPSCs) can successfully be differentiated into cardiomyocytes (iPSC-CMs) [1], creating a promising supply of iPSC-CMs to regenerate the heart post-infarction or for use in disease modeling and drug screening applications [2-4]. However, iPSC-CMs are limited by their large batch-to-batch variability in differentiation efficiencies, which may be caused in part by heterogeneity in the traditional stem cell culture substrate Matrigel [5,6]. Matrigel consists of basement-membrane extracellular matrix (ECM) proteins and growth factors derived from an Englebreth-Holm-Swarm tumor formed in mice. This substrate can lead to xenogeneic contamination of cells during culture [6] and has variable composition between batches [7-9], resulting in unpredictable cell behavior [3,10]. In addition, the Matrigel shear modulus has ranged from 0.1–0.3 kPa, whereas cardiac tissue ranges from 0.5–6.0 kPa throughout heart development and can reach greater than 10 kPa during cardiac disease [4,5,11,12].

Synthetic hydrogel substrates have emerged as an alternative to Matrigel for cell culture applications [6]. For example, poly(ethylene glycol) (PEG) hydrogels are widely used as a base substrate, as they are typically bioinert, but can be readily modified to present cell adhesion ligands. Synthetic hydrogels have often been used as an experimental tool to systematically study the influence of a specific substrate chemical or mechanical property on cell behavior [13,14]. Simultaneous co-variation of multiple substrate properties has been more rarely explored, but is important to ascertain how multiple variables interact synergistically to influence a cell behavior of interest. In

particular, studying multi-variate effects of substrate properties on iPSC-CM differentiation may identify substrate formulations that effectively improve upon and replace Matrigel, and maximize development of a desired cell phenotype.

Here, we hypothesized that systematically and combinatorially varying multiple synthetic ECM properties would identify biomaterials that support adhesion, survival, and differentiation of iPSC-derived cardiac progenitor cells (iPSC-CPCs) to cardiomyocytes. The ranges of substrate parameters used here were selected to mimic aspects of human cardiac development, including stiffness ranges and prevalent types of cell adhesion ligands [15]. Our screening approach identified PEG hydrogel formulations able to support adhesion and survival of iPSC-CPCs, and showed greater iPSC-CM differentiation efficiency and reproducibility than the gold standard Matrigel substrate.

2.3 Materials and Methods

2.3.1 iPSC Culture and Maintenance

WTC11 human iPSCs were maintained on 6-well plates coated with Matrigel (Corning) in mTeSR medium (Stem Cell Technologies) at 37°C and 5% CO₂. The media was changed every day and cells were passaged every 3 days using Versene (Life Technologies) for transfer to fresh Matrigel-coated plates.

2.3.2 iPSC-Derived Cardiomyocyte Differentiation

iPSCs were differentiated to cardiomyocytes as previously described [16]. Briefly, stem cells were passaged to a fresh Matrigel-coated 12-well plate using Accutase (Sigma Aldrich) and seeded at 40,000 cells/cm² in mTeSR medium with 5 uM ROCK inhibitor (R&D Systems). Cells were expanded for 2 days with daily changes of mTeSR medium.

On day 0 of differentiation, the media was changed to RPMI 1640 medium (Thermo Fisher Scientific) with B27 supplement without insulin (B27-; Thermo Fisher Scientific), supplemented with 7 μ M CHIR-99021 (Selleck Chemicals), to induce differentiation. After 48 hours, cells were fed with fresh RPMI 1640 medium with B27- supplemented with 5 μ M IWP2 (Tocris). After an additional 48 hours, cells were then fed with fresh RPMI 1640 medium with B27-. On day 6 of differentiation, the media was replaced with fresh RPMI 1640 medium with B27 supplement with insulin (B27+; Thermo Fisher Scientific). Cells were then fed with RPMI 1640 medium with B27+ every 2-3 days until day 16 of differentiation. Initial steps of the differentiation were conducted on Matrigel until the cardiac progenitor stage (day 5 of differentiation), at which point the cells were lifted and singularized using Accutase and frozen in liquid nitrogen. Cells were then thawed and seeded either on Matrigel or PEG hydrogels for continued differentiation.

2.3.3 PEG Hydrogel Synthesis

PEG hydrogels were prepared using 8-arm, 20 kDa norbornene-functionalized PEG (JenKem Technology), 3.4 kDa PEG di-thiol (Laysan Bio) crosslinker, 0.1 wt/wt% Irgacure 2959 (CIBA) photoinitiator, diluted with DPBS (Gibco) to reach desired concentrations. The following peptides were added at varying concentrations: CRGDSP (3.5 mM or 7 mM) or head-to-tail cyclized RGDfC (cRGDfC; 3.5 mM or 7 mM), referred to as a primary peptide, and CIKVAV (1.5 mM or 3 mM) or CYIGSR (1.5 mM or 3 mM), referred to as a secondary peptide. All peptides were purchased from GenScript. The shear modulus of the hydrogels was varied by adjusting the monomer-to-crosslinker ratio. We assayed physiologically relevant stiffnesses, measured as shear modulus,

corresponding to neonatal (0.5 kPa and 2 kPa), healthy adult (4 kPa and 6 kPa), and fibrotic (10 kPa) cardiac tissue [4,5,11,12]. A full factorial design of these 3 variables (primary peptide, secondary peptide, and shear modulus) was used to define the hydrogel formulations tested here.

Hydrogel shear modulus was characterized using methods previously described [17-20]. Shear modulus was tested on bulk hydrogel samples demonstrating dimensions of 8.0 mm diameter and 1.2 mm depth. Hydrogels were incubated in PBS for 24 hours to allow swelling prior to testing. The Ares-LS2 rheometer (TA Instruments) was used to test hydrogel shear modulus. Parallel plates of the same dimensions as our hydrogel samples were used to apply a 20 g force. A dynamic strain sweep test with 1 Hz frequency was performed between 0.1 and 50 % strain. The shear modulus for each sample was measured between 1 to 10 % strain.

Peptide incorporation into the hydrogel during polymerization was assessed using methods previously described [17-20]. Briefly, the availability of free thiol groups was measured using the Ellman's assay (Thermo Fisher Scientific). After hydrogel polymerization, the gels were incubated in Ellman's buffer for 20 minutes. Absorbance was read on a plate reader at 412 nm. A standard curve using L-Cysteine hydrochloride was used to measure remaining thiol content in the hydrogel.

2.3.4 Confluency Characterization

Cells were imaged on day 3 after seeding to assess confluency levels. Samples were stained by washing with PBS and incubating in RPMI 1640 medium with B27+, containing 1.5 uM ethidium homodimer-1 and 1 uM calcein-AM (Thermo Fisher

Scientific), for 30 minutes at 37°C. Samples were imaged on a Nikon TI Eclipse microscope. Samples were washed twice with PBS before adding fresh RPMI 1640 medium with B27+ supplement, for continued differentiation. Using FIJI, confluency levels were determined by thresholding fluorescence intensity and quantifying total object area per sample.

2.3.5 Flow Cytometry

Cardiomyocyte purity was assessed using flow cytometry to measure cardiac troponin T (cTnT) expression for cells after 11 days of culture. Cells were lifted and singularized with Accutase before transferring to tubes containing DMEM/F12 medium (Thermo Fisher Scientific). Samples were centrifuged at 200xg for 5 minutes and cells were resuspended in 1% formaldehyde solution and incubated in the dark for 20 minutes at room temperature. Samples were centrifuged and resuspended in 90% methanol solution in the dark for 20 minutes at 4°C before being transferred to -20°C until processing. Samples were washed twice in Flow Buffer 1 (PBS containing 0.5 % BSA) and incubated overnight at 4°C in Flow Buffer 2 (PBS containing 0.5% BSA and 0.1% Triton X-100) with a primary antibody. Samples were then washed with Flow Buffer 2, incubated for 30 minutes at room temperature in the dark in Flow Buffer 2 with a secondary antibody, washed with Flow Buffer 2, and resuspended in Flow Buffer 1. Data was collected on an Attune flow cytometer (Thermo Fisher Scientific) and analyzed on FCS Express software. The primary antibodies used were mouse anti-human cardiac troponin T (cTnT; Abcam, 1:800), mouse anti-human ISL-1 (DSHB, 1:20), and goat anti-human NKX2.5 (Abcam, 1:100). The secondary antibodies used were donkey anti-mouse

AlexaFluor 488 (Thermo Fisher Scientific, 1:1000) and donkey anti-rabbit AlexaFluor 647 (Thermo Fisher Scientific, 1:1000).

2.3.6 Immunofluorescence Imaging

Images of cardiomyocytes were taken after 11 days in culture. Briefly, cells were fixed by incubating in 4 % formaldehyde solution for 20 minutes in the dark. After washing, fresh PBS was added and samples were placed at 4°C until staining. Samples were incubated in Flow Buffer 2 for 1 hour at room temperature, followed by an overnight incubation in Flow Buffer 2 with primary antibodies at 4°C. The samples were washed with PBS three times for 10 minutes before incubating in Flow Buffer 2 with secondary antibodies for 1 hour in the dark at room temperature. The samples were then washed an additional three times for 10 minutes before adding fresh PBS and placing samples at 4°C until imaging. Images were taken using the TI Eclipse microscope (Nikon). The primary antibody used was mouse anti-human cTnT (Abcam, 1:200). The secondary antibody used was donkey anti-mouse AlexaFluor 488 (1:1000). DAPI was used for nuclear staining (Thermo Fisher Scientific, 1:1000).

2.3.7 Data Analysis and Statistics

Multi-variate analysis was performed using JMP statistical analysis software. A standard least squares model was generated and Student t-test was performed to assess the effect of each culture parameter on the measured outcome (e.g. cell confluency, differentiation efficiency). Additional statistical analysis was performed using GraphPad Prism software with Student t-test or one-way ANOVA as indicated.

2.4 Results

2.4.1 Synthetic PEG Hydrogels Enabled Control of Substrate Properties

A three-factor, five-level full factorial design was used to define the PEG hydrogel conditions which were screened for iPSC-CPC adhesion, survival, and differentiation (Figure 2.1A). Using a fully defined hydrogel synthesis workflow, we developed PEG hydrogels with varying formulations (Figure 2.1B, Supplementary Table 2.1). By manipulating the monomer-to-crosslinker ratio, we observed a high level of control over the resulting hydrogel shear modulus, with moduli ranging from 0.5 kPa (2.5 mM PEG-NB, 10 mM PEG-DT) to 10 kPa (8 mM PEG-NB, 64 mM PEG-DT). The hydrogel moduli spanned the ranges measured in previous studies for cardiac tissue at different developmental stages: < 3 kPa in a neonatal environment, 3 – 9 kPa in a healthy environment, and > 9 kPa in a fibrotic environment [15,21-24] (Supplementary Figure 2.1A). In addition, synthetic peptides were efficiently incorporated into the PEG hydrogels, ranging between 85 – 95 % thiols reacted across conditions (Supplementary Figure 2.1B). The resulting hydrogel swelling ratio from preparation to fully swollen varied with respect to the concentration of input materials, ranging between 11 – 27 % swelling across conditions (Supplementary Figure 2.2).

2.4.2 Synthetic PEG Hydrogels Supported iPSC-Derived Cardiac Progenitor Cell Adhesion and Survival

Screening 125 PEG hydrogel formulations identified 52 PEG hydrogels that supported iPSC-derived cardiac progenitor cell (iPSC-CPC) adhesion and survival (Figure 2.2), defined here as greater than 20 % cell confluency, at 72 hours post-seeding.

A three-variable, five-level full factorial design generated 125 initial PEG hydrogel formulations that were tested for their ability to support iPSC-CPC adhesion and survival. iPSC-CPC populations adopted distinct morphologies on different PEG hydrogel surfaces, ranging from large cell colonies to dispersed cell clusters (Figure 2.2A). This observation also translated to varying confluency levels across hydrogel conditions (Figure 2.2B). All PEG hydrogel conditions without an RGD peptide were unable to support cell survival, indicating that the laminin-derived peptides (IKVAV, YIGSR) alone did not support iPSC-CPC adhesion and survival at the ligand densities tested (Figure 2.2C). The extent of cell dispersion on the PEG hydrogel surface also varied with the identity of the synthetic peptide (Figure 2.2C, Supplementary Figure 2.3). The presence of c(RGDfC) resulted in higher cell confluency (Supplementary Figure 2.3A), reaching up to 50 % for the PEG hydrogel formulation 3.5 mM c(RGDfC) at 0.5 kPa (Figure 2.2B). Cell confluency levels were also greater on substrates with lower stiffnesses, with moduli similar to those present in neonatal and healthy adult cardiac tissues (Figure 2.2D). In contrast, PEG hydrogels with the highest shear modulus tested (10 kPa) had limited cell survival at 72 hours post-seeding (Figure 2.2D). iPSC-CPCs on Matrigel were highly confluent, reaching 73 ± 5 % confluency, surpassing levels seen on PEG hydrogel formulations.

2.4.3 Properties of Synthetic PEG Hydrogels Individually Influenced iPSC-Derived Cardiac Progenitor Cell Confluency

A multi-variate analysis revealed key hydrogel variables that influenced iPSC-CPC confluency levels, with a coefficient of determination of 0.43 (Supplementary Figure 4A).

Both c(RGDfC) and CRGDSP peptides had a direct effect on the level of cell confluency ($p < 0.0001$ and $p < 0.01$, respectively). Higher concentrations of these two peptides resulted in greater cell confluency (Figure 2.2E). Hydrogel stiffness also had a significant negative correlation with iPSC-CPC confluency ($p < 0.001$, Figure 2.2E). However, the interactive effects of multiple substrate variables did not significantly influence cell confluency levels (Figure 2.2E).

2.4.4 Synthetic PEG Hydrogels Supported Robust Levels of iPSC-Derived Cardiac Progenitor Cell Differentiation into cTnT+ iPSC-Derived Cardiomyocytes

PEG hydrogels supported high levels of iPSC-CPC differentiation into iPSC-CMs, regardless of substrate formulation. Due to the large number of samples, one third of the hydrogel samples (48 out of 126 conditions, with 3 replicates each) were initially selected at random to assess the overall level of differentiation (Supplementary Table 2.2). Synthetic hydrogels supported high levels of differentiation, as 74 ± 5 % of cells on synthetic hydrogels expressed the cardiomyocyte marker cTnT (Figure 2.3A). In addition, iPSC-CMs differentiated on PEG hydrogels exhibited apparently high levels of cTnT expression across the entire surface of the substrate (Figure 2.3C), with robust, uniform contractility (Supplementary Figure 2.5). In comparison, 60 ± 11 % of cells differentiated on Matrigel expressed cTnT at an apparently lower level than the cells on PEG hydrogels (Figure 2.3A-B). Further, no difference was observed across the CM markers at different steps in the differentiation (progenitor stage: NKX2.5, ISL1; differentiated stage: cTnT) for cells differentiated on PEG hydrogels, whereas we observed a significant decrease in the cell population expressing ISL1, a cardiac progenitor marker, and that expressing

cTnT, a cardiomyocyte marker, for cells differentiated on Matrigel (Figure 2.3C, Supplementary Figure 2.6, Supplementary Table 2.3).

In a separate experiment, we evaluated whether PEG hydrogel substrates could “rescue” a differentiation – that is, whether PEG hydrogels could improve differentiation of iPSC-CPC populations that showed poor differentiation efficiency on Matrigel. In particular, a batch of iPSC-CPCs that showed 36 ± 3 % cTnT expression when differentiated on Matrigel in one experiment, was re-seeded onto various PEG hydrogel formulations in a second experiment (Supplementary Table 2.4). On the PEG hydrogels, the cells reached 52 ± 6 % cTnT expression across all formulations, with the best performing hydrogel formulation supporting 62 ± 12 % iPSC-CM differentiation (Figure 2.3D). In comparison, iPSC-CPCs reseeded onto Matrigel in the second experiment reached only 40 ± 10 % cTnT expression (Figure 2.3D).

2.4.5 PEG Hydrogel Parameters Influenced iPSC-Derived Cardiac Progenitor Cell Differentiation into iPSC-Derived Cardiomyocytes

The identity of cell adhesion peptides present in the PEG hydrogel substrates significantly influenced iPSC-CPC differentiation into iPSC-CMs (Figure 2.4A). A multivariate analysis model was developed, with a coefficient of determination of 0.56 (Supplementary Figure 2.4B), to study the roles of these substrate parameters in iPSC-CM differentiation efficiency. The primary peptide (either c(RGDfC) or CRGDSP) correlated positively with cTnT expression ($p < 0.0001$), indicating a strong influence of the c(RGDfC) peptide (Figure 2.4B). Similarly, the secondary peptide (either CIKVAV or CYIGSR) correlated positively with iPSC-CPC differentiation into iPSC-CMs ($p < 0.01$),

highlighting the importance of the CYIGSR peptide (Figure 2.4B). The interactive effects of multiple substrate variables did not demonstrate a significant influence on differentiation efficiency (Figure 2.4B). In addition, hydrogel stiffness, measured as shear modulus, did not have a significant influence on iPSC-CM differentiation over the range tested here (Figure 2.4B). Further, an additional multi-variate analysis model, with a coefficient of determination of 0.39 (Supplementary Figure 2.4C), revealed the influence of both the cell number and cell confluency levels on differentiation efficiency. Both cell number and confluency level were observed to correlate positively with iPSC-CM differentiation levels ($p = 0.001$ and $p = 0.01$, respectively) (Figure 2.4C), highlighting the influence of both cell-cell and cell-substrate interactions on iPSC-CM differentiation.

2.4.6 Synthetic PEG Hydrogels Support Reproducible Levels of iPSC-Derived Cardiac Progenitor Cell Differentiation into iPSC-Derived Cardiomyocytes

iPSC-CPC differentiation levels were more reproducible on PEG hydrogels between experiments and between experimental replicates when compared to iPSC-CPC differentiation on Matrigel. Across experiments, PEG hydrogels supported a higher rate of reproducibility, measured as the standard deviation of % cTnT expression, compared to Matrigel (Figure 2.5A). The reproducibility level on PEG hydrogels was measured as an average of 48 different PEG formulations (Supplementary Table 2.2), highlighting that the platform itself supported reproducible differentiation levels irrespective of the specific formulation. Similarly, the PEG hydrogels supported superior reproducibility to Matrigel across replicates (Figure 2.5B). This highlighted the heterogeneity of Matrigel even within

a single batch, as previously reported [7,8], and the relative consistency of the synthetic PEG formulations.

2.5 Discussion

Synthetic materials have proven effective in improving differentiation outcomes for several cell types, including hMSCs [25,26], endothelial cells [20,25], hepatoblasts [27,28], neural stem cells [29], iPSC-derived alveolar cells [30], and PSC-CMs [16], and outperforming the traditional Matrigel substrate. The complex and variable composition of Matrigel has had a direct influence on stem cell behavior, including on stem cell-derived cardiomyocyte differentiation [31-33]. Proteins found in Matrigel (e.g. Matrix metalloproteinase-3 and Chemokine ligand 2) and their negative influence on stem cell differentiation may contribute to limited iPSC-CM differentiation efficiency [31]. Through simultaneous variation of multiple substrate properties, our work builds upon other studies showing that synthetic materials improve iPSC-CM differentiation outcomes when compared to Matrigel [34-36]. For example, Jung et al. performed an engineering optimization to develop PEG hydrogels with full-length ECM proteins for improved iPSC-CM differentiation efficiency [37]. They demonstrated that a higher fraction of cells expressed the cardiomyocyte marker cTnT when cultured on their optimized substrate (40 mg/mL PEG and 1.15, 0.45, and 0.28 mg/mL of collagen, laminin, and fibronectin, respectively) when compared to blank PEG and PEG hydrogel crosslinked with the study's maximum concentration of each ECM protein [37]. Here, by systematic co-variation of multiple ECM properties, with ranges of ECM properties restricted to mimic some aspects of human cardiac development, we identified PEG hydrogel conditions

relevant for iPSC-CM differentiation and compared our material to Matrigel, the traditional substrate in iPSC-CM differentiations [16]. Further, we observed a clear increase in iPSC-CM differentiation efficiency across all the PEG hydrogel formulations (Figure 2.3A), highlighting the direct advantage of using synthetic PEG hydrogels relative to Matrigel.

Although high efficiency iPSC-CM differentiation is achievable via traditional methods (i.e. culture on the Matrigel substrate or other ECM-derived proteins) [1,16], differentiation efficiencies are highly variable across replicates and batches [38]. The variability in differentiation efficiencies is often attributed to the complexity and lack of compositional reproducibility in the Matrigel substrate, despite extensive quality testing required of this material to validate its ability to support iPSC culture and CM differentiation [6,9]. Notably, Matrigel mechanical and biochemical properties have been highlighted for their lack of reproducibility, as seen through highly variable elastic modulus [8] and protein composition [31]. Matrigel variability may directly translate into variability in cell behavior, including in cell differentiation [31,39]. We have reasoned that synthetic materials, which are fully defined and tunable, could potentially minimize the variability in differentiation outcomes. Extensive characterization and use of fully-defined components in synthetic materials has been shown to contribute to superior reproducibility of other cell culture results [6,40]. For instance, a previous study showed greater reproducibility of endothelial network formation by HUVECs and iPSC-derived endothelial cells when using PEG-based hydrogels when compared to Matrigel [20]. Reproducible endothelial network formation also led to superior sensitivity and reproducibility of an endothelial “tubulogenesis” assay commonly used to evaluate angiogenesis regulators [20]. In the

current study we used the same materials, at different ratios, to achieve greater reproducibility in iPSC-CM differentiation efficiency on PEG-based hydrogels (Figure 2.5). Specifically, we observed a 44 % decrease in variability of cTnT expression across experimental replicates of PEG hydrogel conditions compared to Matrigel and, similarly, a 68 % decrease in variability across experiments (Figure 2.5). This suggests that these cells could also have greater value in cardiac disease modeling.

By varying multiple substrate properties simultaneously, we observed the emergence of synergies between the effects of different hydrogel properties. Although our PEG hydrogels broadly outperformed Matrigel regardless of the formulation, multivariate analysis revealed the significant influence of the fibronectin- and laminin-derived peptides on iPSC-CM differentiation (Figure 2.4B). This observation aligns with previous studies highlighting the importance of multiple ECM proteins, such as collagen, laminin, and fibronectin, in iPSC-CM differentiation [36,41,42]. For example, the significance of laminin in iPSC-CM differentiation was observed in a previous multivariate model [37]. Jung et al. showed that the ECM proteins studied were positive in maximizing cTnT expression [37], as was the case in our multivariate model showing a positive correlation between cTnT expression and the synthetic cell adhesion peptides tested (Figure 2.4B). Further, Jung et al. highlighted that laminin had the most significant main effect on differentiation when compared to collagen and fibronectin [37]. In comparison, we showed that the c(RGDfC) and CRGDSP peptides have an integral role in the iPSC-derived CPC-to-CM differentiation (Figure 2.4B), possibly indicating that the role of the RGD peptide motif (fibronectin-derived) is greater during terminal differentiation from progenitor to

cardiomyocyte. Neiman et al. showed increased expression of fibronectin at the CPC stage, compared to the undifferentiated stage. The cell integrin receptor subunit $\alpha 5$ engaged by fibronectin was also shown to be up-regulated at the CPC stage [43]. Similarly as in our work, fibronectin appeared to have a crucial role at the CPC stage of iPSC-CM differentiation [43]. Further, we showed the importance of c(RGDfC) and CRGDSP peptides for cell confluency (Figure 2.2E). The increased level of cell confluency may have contributed to improvements in iPSC-CM differentiation. Although substrate stiffness, measured as the shear modulus, has been previously shown to influence iPSC-CM differentiation [10,11], it had a minimal influence here in comparison to the influence of ECM-derived cell adhesion peptides (Figure 2.4B). This may be caused by the relatively small range of shear modulus assessed here, as substrate stiffness can be varied over a much larger range of moduli (100s of kPa) [40] but was limited here in order to remain within physiologically relevant cardiac stiffnesses [7,8,10,11]. Cell adhesion ligands played an apparently more significant role in iPSC-CPC differentiation into iPSC-CMs compared to substrate stiffness (Figure 2.4B).

We showed that individual substrate variables directly influenced iPSC-CM differentiation efficiency whereas combinatorial effects were not significant (Figure 2.4B). This highlights the apparently independent roles of substrate stiffness and cell adhesion ligands on iPSC-CPC differentiation into iPSC-CMs in the context of our experimental parameter space. The individual influence of fibronectin and laminin proteins was previously observed by Jung et al., and their combination demonstrated a lesser effect [37]. Specifically, collagen and laminin had a significant influence on expression of the

cardiomyocyte marker cTnT, with fibronectin having a less significant role, while all interaction effects between distinct proteins did not significantly influence cTnT expression. Here, we similarly showed a stronger influence of individual substrate variables when compared to interactive effects (Figure 2.4B).

2.6 Conclusion

Efficient manufacturing of human iPSC-derived cells is highly dependent on the cell culture substrate and its ability to support effective and reproducible differentiation. Here, we identified fully-defined, xeno-free, PEG-based hydrogels as an alternative to Matrigel for human iPSC-CM differentiation. The tunability of this technology offered a range of formulations that supported iPSC-CPC survival and iPSC-CM differentiation levels that outperformed those seen on Matrigel, while also providing significantly enhanced reproducibility. The screening approach employed here is generalizable to other cell types and could aid in identifying key environmental variables for specific human stem cell applications.

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2.8 References

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2.9 Figures

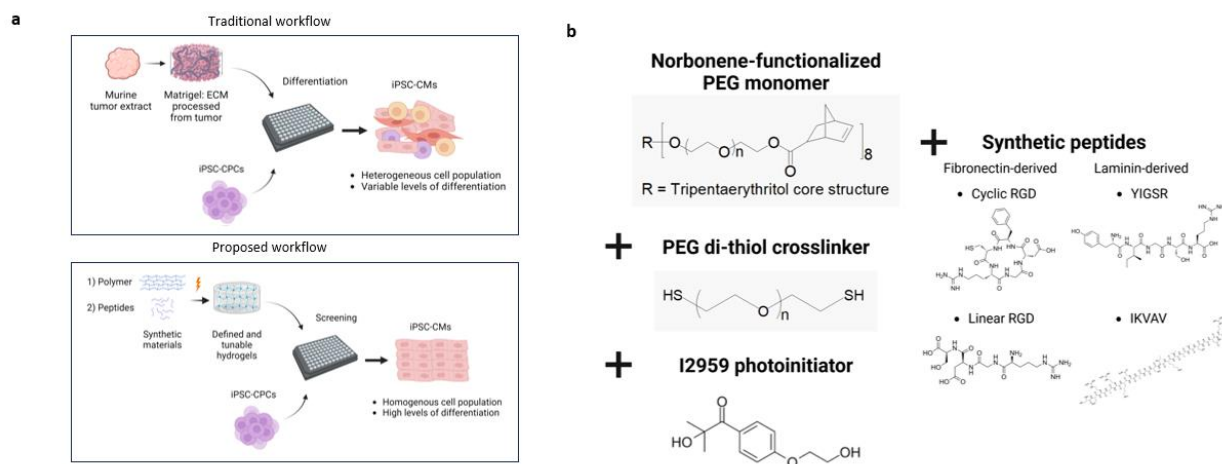


Figure 2.1. Schematic overview of Experiments

Schematic representation of **(A)** the traditional workflow and our proposed workflow relying on screening of PEG hydrogel formulations, and **(B)** the PEG hydrogel synthesis workflow. Schematic not to scale.

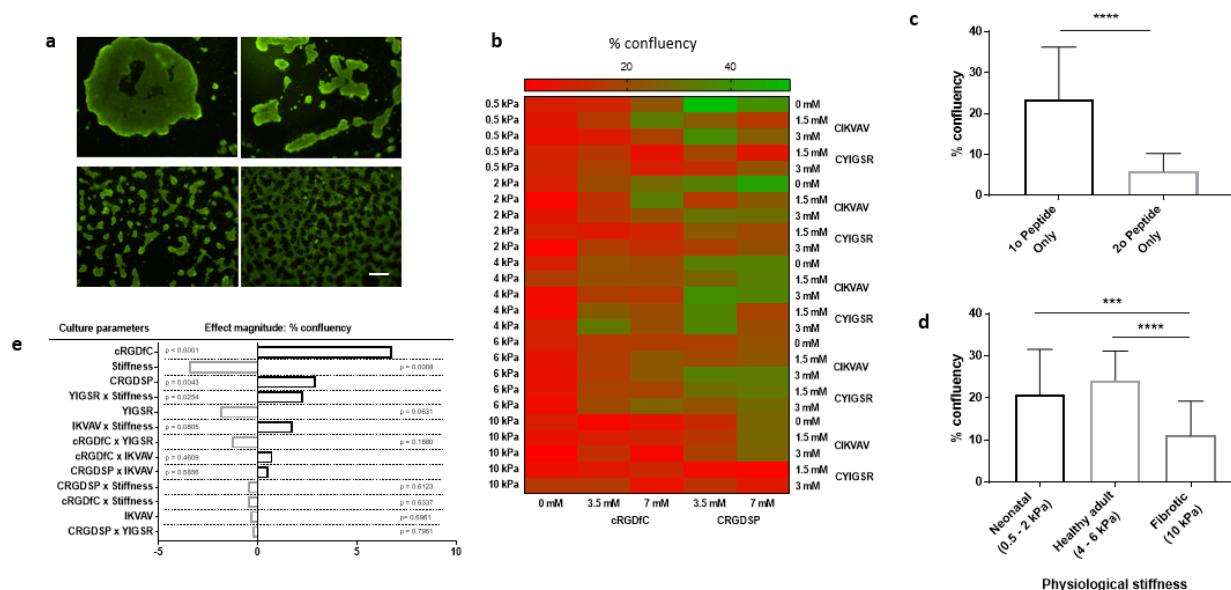


Figure 2.2. Synthetic PEG hydrogels, representative of developmental stages of the cardiac ECM, supported iPSC-CPC adhesion and survival.

(A) Representative calcein-AM fluorescence images of live iPSC-CPCs cultured on PEG hydrogels 3 days after seeding. Scale bar = 500 μ m. (B) Quantitative heat map of iPSC-CPC confluency levels at 72 hours post-seeding on PEG hydrogels. Each box = 3 replicate gels. (C) Confluency quantification of iPSC-CPCs cultured on PEG hydrogels presenting different peptides. **** $p < 0.0001$, Student t-test. Each bar = 25 gels. (D) Confluency quantification of iPSC-CPCs cultured on PEG hydrogels with different stiffnesses. *** $p < 0.001$, one-way ANOVA. Each bar = 50 gels (neonatal & healthy adult) or 25 gels (fibrotic). (E) Multivariate analysis (MVA) of individual and interactive effects of hydrogel variables on iPSC-CPC confluency levels.

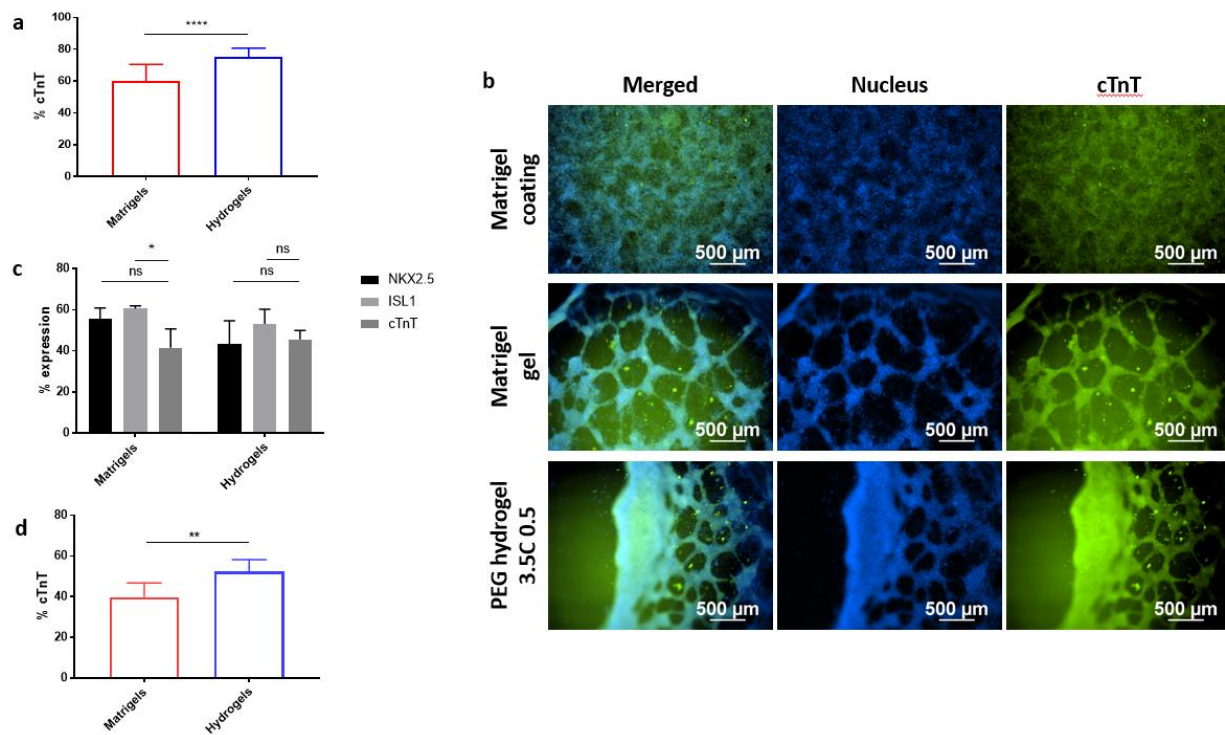


Figure 2.3. PEG hydrogels supported high levels of iPSC-CM differentiation.

(A) Flow cytometry quantification of % cTnT-expressing cells with terminal differentiation conducted on Matrigel or PEG hydrogels. **** $p < 0.0001$, Student t-test. Each bar = 9 gels (Matrigel) or 126 gels (PEG hydrogels). (B) Flow cytometry quantification of CPC markers (NKX2.5, ISL1) and CM markers (cTnT) on Matrigel and PEG hydrogels. ns: no significance, * $p < 0.05$, one-way ANOVA. Each bar = 6 gels (Matrigel) or 9 gels (PEG hydrogels). (C) Immunofluorescence images of iPSC-CMs on different substrates. (D) Flow cytometry quantification of % cTnT-expressing cells rescued for terminal differentiation on PEG hydrogels or Matrigel. ** $p < 0.01$, Student t-test. Each bar = 9 gels (Matrigel) or 44 gels (PEG hydrogels). Scale bar = 500 μ m.

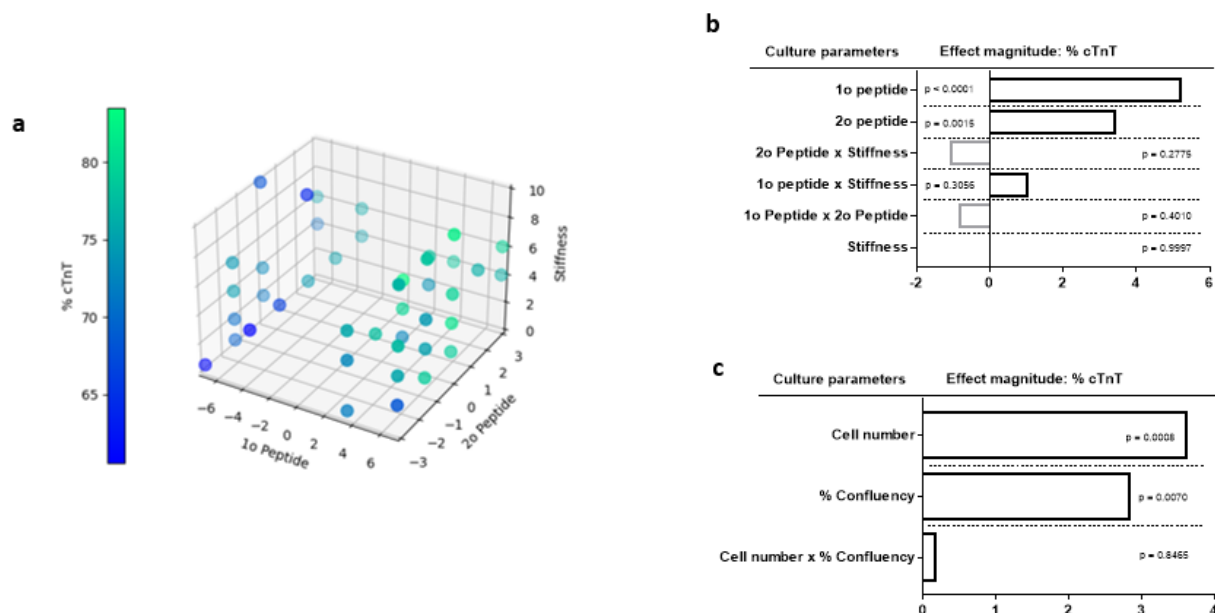


Figure 2.4. PEG hydrogel formulations influenced iPSC-CM differentiation efficiency.

(A) 3D plot representative of % cTnT-expressing cells in PEG hydrogel formulations of varying primary peptide (0-7 mM CRGDSP, represented as negative values, or 0-7 mM c(RGDfC)), secondary peptide (0-3 mM CIKVAV, represented as negative values, or 0-3 mM CYIGSR), and stiffness (0.5-10 kPa). Each dot = 3 replicate gels. **(B)** MVA of individual and interactive effects of hydrogel variables on iPSC-CM differentiation levels. **(C)** MVA of cell number and confluency level effects on iPSC-CM differentiation levels.

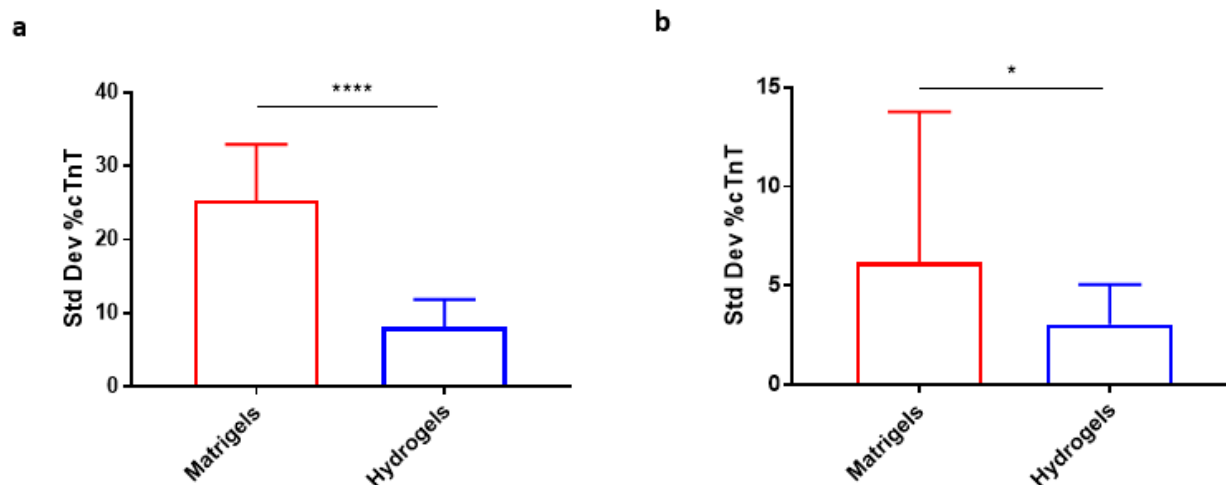
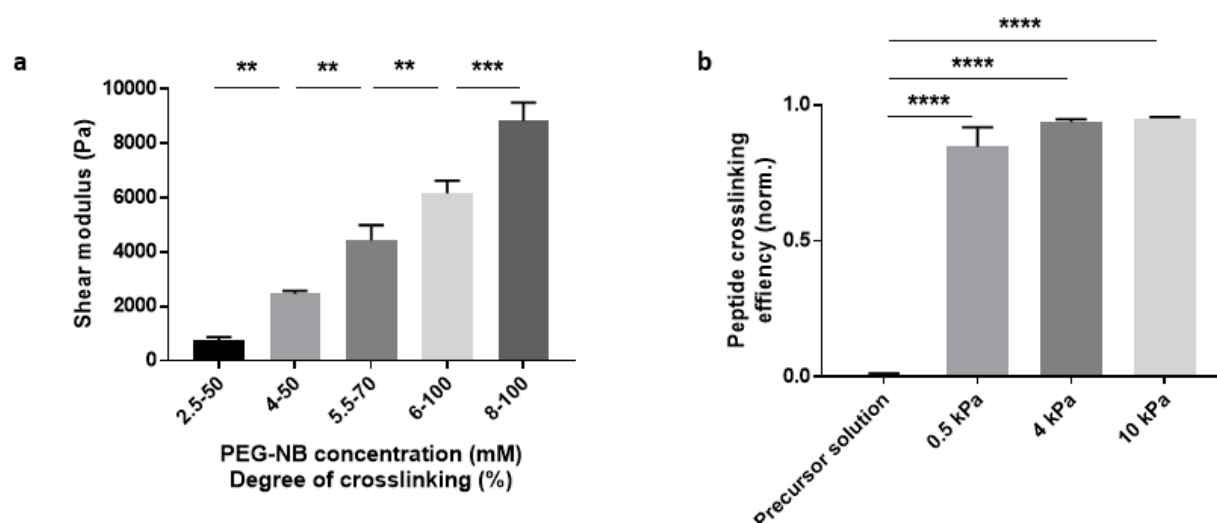


Figure 2.5. Reproducibility of iPSC-CM differentiation efficiency.

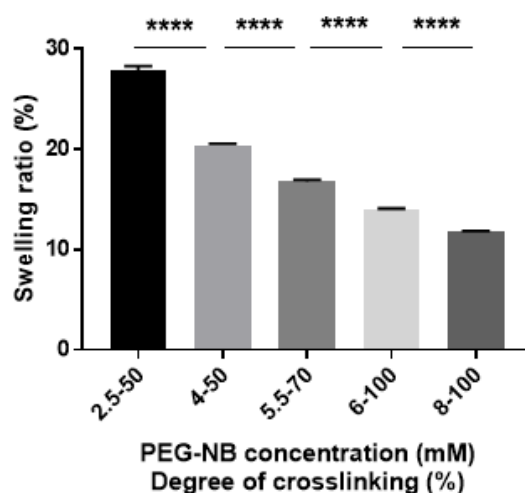
PEG hydrogels supported superior reproducibility in iPSC-CM differentiation efficiencies when compared to Matrigel **(A)** between experiments, **** $p < 0.0001$, Student t-test, each bar = 3 experiments for 3 conditions (Matrigel) or 42 conditions (PEG hydrogels); and **(B)** between experimental replicates, * $p < 0.05$, Student t-test, each bar = 3 replicates for 3 conditions (Matrigel) or 42 conditions (PEG hydrogels).

2.10 Supplemental Figures



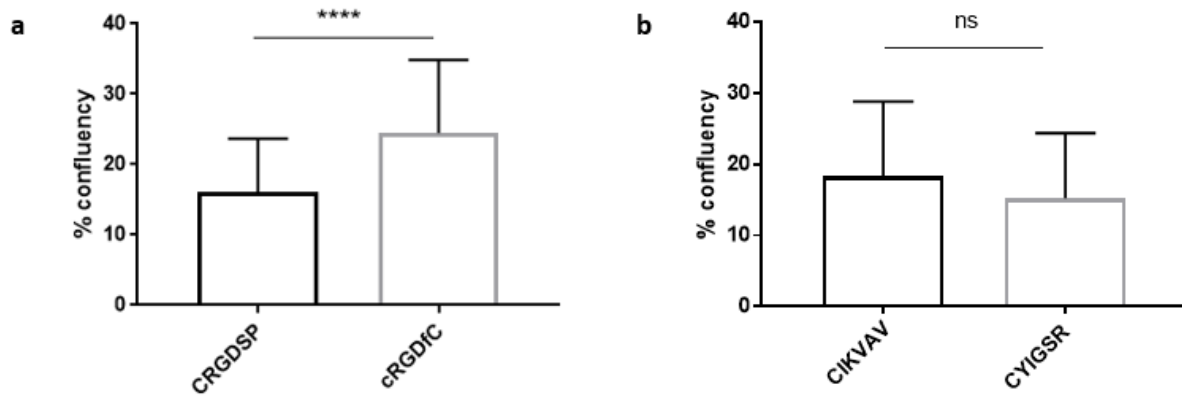
Supplementary Figure 2.1 Characterization of PEG hydrogel properties.

PEG hydrogel characterization revealed precise control of **(A)** substrate stiffness, measured as shear modulus, and **(B)** peptide concentration. ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$, one-way ANOVA. Each bar = 3 replicate gels.



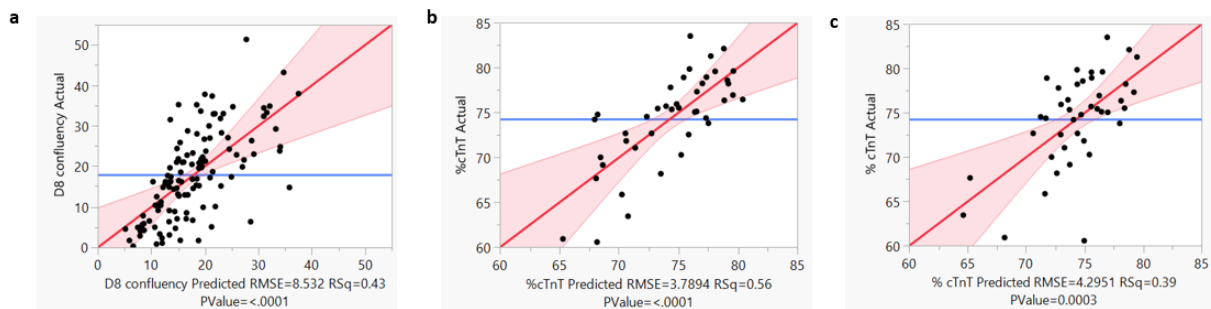
Supplementary Figure 2.2. Characterization of PEG hydrogel swelling ratio.

PEG hydrogel swelling ratio varied with input concentrations. **** $p < 0.0001$, one-way ANOVA. Each bar = 3 replicate gels.



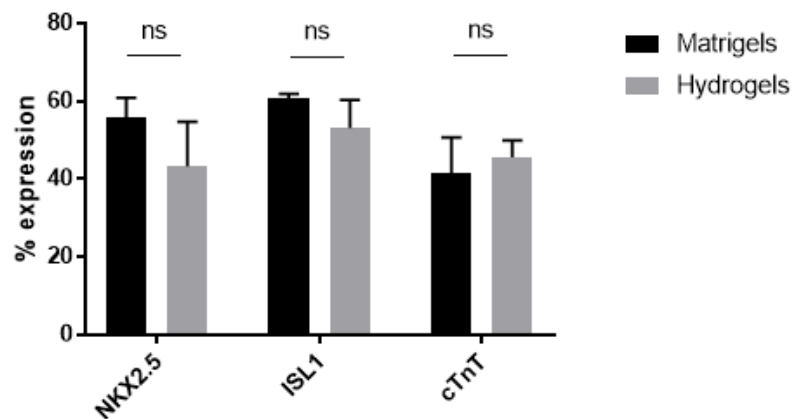
Supplementary Figure 2.3. Cell confluency on PEG hydrogels demonstrating various adhesion ligands.

Confluency quantification of iPSC-CPCs cultured on PEG hydrogels presenting (A) different primary peptides, and (B) different secondary peptides. ns: no significance, **** $p < 0.0001$, Student t-test. Each bar = 50 gels.



Supplementary Figure 2.4. Multi-variate analysis.

Actual by predicted plots of standard least squares models of the effect of (A) substrate parameters on the degree of cell confluency (Figure 2.2E), (B) substrate parameters on the level of cTnT expression (Figure 2.4B), and (C) confluency levels and cell number on the level of cTnT expression (Figure 2.4C).



Supplementary Figure 2.5. Expression of iPSC-CM differentiation markers.

Flow cytometry quantification of CPC markers (NKX2.5, ISL1) and CM markers (cTnT) on Matrigel and PEG hydrogels. ns = no significance, one-way ANOVA. Each bar = 6 gels (Matrigel) or 9 gels (PEG hydrogels).

2.11 Supplemental Tables

Synthetic peptide	Primary/Secondary	Abbreviation	ECM protein
CRGDSP	Primary	L	Fibronectin
head-to-tail cyclized RGDfC	Primary	C	Fibronectin
CIKVAV	Secondary	I	Laminin
CYIGSR	Secondary	Y	Laminin

Supplemental Table 2.1. Description of synthetic peptides incorporated in PEG hydrogels.

Primary peptide	Concentration (mM)	Secondary peptide	Concentration (mM)	Shear modulus (kPa)
L	7	-	-	0.5
L	7	-	-	2
L	3.5	-	-	4
L	7	-	-	4
L	3.5	-	-	10
L	7	-	-	10
C	3.5	-	-	0.5
C	3.5	-	-	2
C	7	-	-	2
C	3.5	-	-	4
C	7	-	-	4
C	3.5	-	-	6
C	7	-	-	6
C	7	-	-	10
L	3.5	I	1.5	4
L	3.5	I	3	4
L	7	I	1.5	0.5
L	7	I	3	0.5
L	7	I	1.5	2
L	7	I	3	2
L	7	I	1.5	4

L	7	I	1.5	6
L	7	I	3	6
L	3.5	Y	1.5	4
L	3.5	Y	3	4
L	3.5	Y	3	6
L	7	Y	3	4
L	7	Y	3	6
C	3.5	I	3	0.5
C	3.5	I	1.5	4
C	3.5	I	3	4
C	3.5	I	3	6
C	7	I	3	0.5
C	7	I	1.5	2
C	7	I	3	2
C	7	I	1.5	4
C	7	I	3	4
C	7	I	1.5	6
C	7	I	3	6
C	7	I	1.5	10
C	7	I	3	10
C	7	Y	3	0.5
C	3.5	Y	1.5	4
C	3.5	Y	3	4
C	3.5	Y	1.5	6
C	7	Y	3	4
C	7	Y	1.5	6
C	7	Y	3	6

Supplemental Table 2.2. PEG hydrogel formulations tested to assess iPSC-CM differentiation efficiency. (Figure 2.3A)

Primary peptide	Concentration (mM)	Secondary peptide	Concentration (mM)	Shear modulus (kPa)
L	3.5	-	-	4
C	3.5	-	-	0.5
C	7	Y	3	6

Supplemental Table 2.3. PEG hydrogel formulations tested to study CPC and CM differentiation markers. (Figure 2.3B)

Primary peptide	Concentration (mM)	Secondary peptide	Concentration (mM)	Shear modulus (kPa)
L	7	-	-	2
L	7	-	-	4
C	3.5	-	-	0.5
C	7	-	-	0.5
C	7	-	-	6
C	7	-	-	10
L	7	I	1.5	6
C	3.5	I	3	0.5
C	3.5	I	3	4
C	3.5	Y	3	0.5
C	7	Y	3	4

Supplemental Table 2.4. PEG hydrogel formulations tested to assess their ability to rescue iPSC-CM differentiation efficiency.

Chapter 3 : Screening Identifies PEG Hydrogel Formulations for iPSC-Derived Cardiomyocyte Differentiation and Maturation

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3.1 Abstract

Efficient cell manufacturing is highly reliant on appropriate cell culture conditions, including the cell culture substrate. Although most cell culture is carried out on Matrigel, a “one size fits all” approach is not ideal for optimal cell manufacturing. Identifying the optimal culture environment has the potential to significantly impact cell manufacturing and downstream applications. Here, we applied an optimization modeling tool to a multi-parametric, multi-screen approach to identify an optimal PEG hydrogel formulation for iPSC-derived cardiomyocyte (iPSC-CM) maturation. We showed the successful application of Bayesian optimization (BO) modeling for efficient screening of a large biomaterial parameter space. We also identified two specific PEG hydrogel formulations that yield greater iPSC-CM maturation than observed on Matrigel. These results highlight the significant advantages of combining screening and modeling to efficiently target a desired cell behavior and propose new material formulations as alternatives to the standard iPSC-CM culture substrate.

3.2 Introduction

Heart disease represents a health and economic burden, affecting 10.6 % of people in the United States in 2017 [1]. The ability to treat heart disease, through the use of cell therapy or drug discovery via disease modeling, is hindered by the limited supply of cardiomyocytes. Cardiomyocytes have limited regenerative capacity as they are limited in their ability to self-renew [2]. The ability to successfully differentiate cardiomyocytes from human induced pluripotent stem cells (iPSC-CMs) opened a number of avenues in heart regeneration, cardiac disease modeling, and drug screening. Although these cells hold great promise, the current number of clinical trials or drug approvals in the cardiac space remains limited [3]. This is in part attributable to the relatively immature state of iPSC-CMs compared to adult cardiomyocytes [4,5].

Multiple approaches have been studied to minimize the gap between iPSC-CMs and adult cardiomyocytes. Successful approaches include 3D cell culture [6,7] and cell transplantation into hearts of animal models [8-10]. Although these approaches have seen considerable success in maturation of iPSC-CMs, they remain limited in their success and in their broad application. Despite successful integration and accelerated maturation, cell transplantation has been noted to cause arrhythmogenicity during PSC-CM integration with the host tissue [8]. PSC-CMs alone form poor tissues in 3D culture and rely on the addition of other cells (e.g. fibroblasts) or electrical pacing setups to form functional tissues, thereby relying on complex cell culture systems that can be difficult to adapt to most applications [6,11]. Additionally, 3D free-floating cell aggregates are limited in their reproducibility due to limited control during aggregate formation and difficulty of

handling [12]. Other studies have looked at traditional 2D culture models combined with an electrical [13] or physical [14] stimulus. These methods have also seen some improvements in iPSC-CM maturation but are limited by the complexity of the system and minimal scalability.

Most studies focus on single variable inputs to improve iPSC-CM maturation, resulting in maturation improvements only in specific aspects of maturation. However, cardiomyocyte maturation is a developmental process that affects the entire cell, with significant changes in morphology and organizational structure, metabolism, and functional abilities [4,5,15]. Synthetic materials offer the ability to reliably control the cell's microenvironment and manipulate multiple variables simultaneously [16,17]. Here, we hypothesized that simultaneous variation of multiple synthetic substrate properties combined with an optimization modeling tool would identify optimized substrate conditions for maximized iPSC-CM maturation in traditional 2D *in vitro* culture. Our screening approach identified two substrate formulations that outperformed the traditional Matrigel substrate by improving iPSC-CM maturation at the structural, metabolic, and functional levels. This work represented a continuation of our results using a similar approach to identify substrate conditions that support iPSC-CM differentiation [18].

3.3 Methods

3.3.1 iPSC Culture and Maintenance

WTC11 iPSCs were maintained on Matrigel-coated plates (Corning) at 37°C and 5% CO₂. Cells were fed with mTeSR medium (Stem Cell Technologies) every day and

passed every 3-4 days by incubating in Versene (Life Technologies) and re-plating on freshly-coated Matrigel plates and fresh mTeSR medium.

3.3.2 iPSC-Derived Cardiomyocyte Differentiation

iPSCs were differentiated to cardiomyocytes as previously described [19]. Briefly, cells were passaged to freshly-coated Matrigel plates by incubating in Accutase (Sigma Aldrich) to singularize before centrifuging and re-plating in mTeSR medium with 5 μ M ROCK inhibitor (R&D Systems) at a density of 40,000 cells/cm². Cells were maintained for 48 hours in mTeSR medium to expand to about 90% confluency. The differentiation was induced (day 0 of differentiation) by feeding cells with RPMI1640 medium (Thermo Fisher Scientific) supplemented with B27 minus insulin (Thermo Fisher Scientific) and 7 μ M CHIR99021 (Selleck Chemicals). After 48 hours, cells were fed with RPMI1640 medium supplemented with B27 minus insulin and 5 μ M IWP2 (Tocris). An additional 48 hours later, the medium was changed to RPMI1640 with B27 minus insulin supplement. iPSC-derived cardiac progenitor cells (iPSC-CPCs) were singularized and frozen 24 hours later (day 5 of the differentiation) in a freezing solution consisting of 60% RPMI1640 medium with B27 minus insulin supplement, 30% FBS (Gibco), and 10% DMSO (Fisher Scientific). The frozen cell solution was placed at -80C overnight and transferred to liquid nitrogen for long-term storage. The remainder of the differentiation was conducted on either Matrigel or PEG hydrogels by re-plating the cells in RPMI1640 medium with B27 minus insulin supplement and 5 μ M ROCK inhibitor. The medium was changed 24 hours later to RPMI1640 medium with B27 supplement (Thermo Fisher Scientific). Cells were

maintained in this medium for the remainder of the differentiation and maturation process with medium changes every 2-3 days (Supplementary Figure 3.1).

For imaging purposes (optical redox imaging, Ca^{2+} fluorescence imaging, MuscleMotion video analysis), iPSC-CPCs were seeded in 96-well angiogenesis plates (Ibidi). For all other analyses (rt-qPCR, flow cytometry, glucose/lactate assays), iPSC-CPCs were cultured in standard 96-well plates (Thermo Fisher Scientific), for greater cell numbers per sample. We validated that there were no cTnT expression differences across the two different plate types (Supplementary Figure 3.2A). In fact, we also observed more accurate scalability in terms of cell number with respect to plate surface area on PEG hydrogels compared to Matrigel substrate, as cell numbers scaled by three-fold on PEG hydrogels and almost 6-fold on Matrigel, for a three-fold increase in surface area (Supplementary Figure 3.2B).

3.3.3 PEG Hydrogel Synthesis

The poly(ethylene glycol) (PEG) hydrogel precursor solutions were developed by combining 8-arm, 20 kDa PEG functionalized with norbonene endgroups (PEG-NB, JenKem Technology), 3.4 kDa PEG functionalized with di-thiol endgroups (PEG-DT, Laysan Bio), and 0.1 wt/wt% Irgacure 2959 photoinitiator (CIBA). The solution was diluted with DPBS (Gibco) to adjust final PEG-NB and PEG-DT concentrations. By varying the PEG-NB and PEG-DT concentrations, we manipulated the shear modulus of the resulting hydrogel. Here, we varied the shear modulus, or stiffness, to mimic different stages of cardiac development, between 0.5 kPa and 10 kPa. We also varied the cell adhesion ligands incorporated into the hydrogels by adding synthetic peptides. For this, we used

CRGDSP or head-to-cyclized RGDfC (cRGDfC), referred to as a primary peptide, and CIKVAV or CYIGSR, referred to as a secondary peptide. The primary peptide concentration varied between 0 – 7 mM and the secondary peptide concentration between 0 – 3.5 mM. All synthetic peptides were purchased from GenScript. The hydrogel shear modulus was characterized via rheological measurements, as previously described [20,21]. Peptide crosslinking was assessed via Ellman's assay as previously described [20]. The three variables described here (primary peptide, secondary peptide, and stiffness) defined our 3-dimensional parameter space.

3.3.4 rt-qPCR

RNA was isolated following instructions from the micro RNeasy isolation kit (Qiagen), on day 30 of iPSC-CM differentiation. RNA purity was measured using a plate reader (Biotek). cDNA synthesis was carried out following instructions from the rt² first strand kit (Qiagen). Gene primers for cTnT, α -MHC, β -MHC, MLC2a, and MLC2v were purchased from Integrated DNA Technologies. The expression level for all genes was normalized to that of EDF1 and DDB1 [22]. Fold change was normalized to the cardiomyocyte marker cTnT gene (TnnT2) expression per sample and to our baseline PEG hydrogel formulation, defined here as 3.5 mM cRGDfC and 0.5 kPa (also referred to as 3.5C 0.5).

3.3.5 Flow Cytometry

Cell internal antigens were assessed by flow cytometry, as previously described [19]. Briefly, cells were singularized by incubating in Accutase (Sigma Aldrich) and transferring to DMEM/F12 medium (Thermo Fisher Scientific). Cells were centrifuged at

200xg for 5 minutes before resuspending in 1 % formaldehyde and incubating at room temperature in the dark for 20 minutes. Cells were centrifuged at 200xg for 5 minutes before resuspending in 90 % methanol and incubating at 4°C for 20 minutes. Cell solution was then placed at -20°C for storage until processing. Samples were washed twice with Flow Buffer 1 (PBS with 0.5 % BSA). Samples were incubated overnight at 4°C in Flow Buffer 2 (PBS with 0.5 % BSA and 0.1 % Triton X-100) with primary antibodies. The samples were washed in Flow Buffer 2 and incubated in Flow Buffer 2 with secondary antibodies at room temperature for 30 minutes. Samples were washed again before resuspending in Flow Buffer 1 for processing. Data was collected on an Attune flow cytometer (Thermo Fisher Scientific) and analyzed on FCS Express software. The primary antibodies used were mouse anti-human cardiac troponin T (cTnT; Abcam, 1:800), mouse anti-human myosin light chain a (MLC2a; Synaptic Systems, 1:200), rabbit anti-human myosin light chain v (MLC2v; ProteinTech, 1:200), and rabbit anti-human cardiac troponin I (cTnI; Abcam, 1:50). The secondary antibodies used were donkey anti-mouse AlexaFluor 488 (Thermo Fisher Scientific, 1:1000) and donkey anti-rabbit AlexaFluor 568 (Thermo Fisher Scientific, 1:1000).

3.3.6 Immunofluorescence Imaging

Cells were stained for immunofluorescence imaging on day 30 of differentiation, as previously described [19]. Briefly, cells were fixed in 4% formaldehyde at room temperature for 20 minutes. Cells were then washed before blocking in Flow Buffer 2 (PBS with 0.5 % BSA and 0.1 % Triton X-100) for 1 hour at room temperature. Samples were then incubated in Flow Buffer 2 with primary antibodies at 4°C overnight. The

samples were washed three times in PBS for 5 minutes prior to incubation in Flow Buffer 2 with secondary antibodies for 1 hour at room temperature. The samples were washed three more times in PBS for 5 minutes before adding fresh PBS. The samples were placed at 4°C until imaging. Images were acquired using a Nikon TI Eclipse microscope with 4x objective lens and analyzed using NIS Elements software (Nikon). The primary antibodies used were mouse anti-human cardiac troponin T (cTnT; Abcam, 1:800) and rabbit anti-human myosin light chain v (MLC2v; ProteinTech, 1:200). The secondary antibodies used were donkey anti-mouse AlexaFluor 488 (Thermo Fisher Scientific, 1:1000) and donkey anti-rabbit AlexaFluor 568 (Thermo Fisher Scientific, 1:1000). Nuclei were stained with DAPI (Thermo Fisher Scientific, 1:1000).

3.3.7 Optical Redox Imaging

On day 30 of differentiation, optical redox imaging was carried out as previously described [23]. All samples were imaged using a Nikon TI Eclipse microscope with 4x objective. Images were collected using exposures of 5 s for NAD(P)H through a standard DAPI filter (460/50 nm) and 2 s for FAD through a standard FITC filter (480/30 nm). One field of view was acquired per sample, with 3 replicate samples per substrate formulation. Images were processed and analyzed using FIJI software, as previously described [1]. Vignetting was corrected by applying a rolling ball subtraction on NAD(P)H images. The images were then thresholded to separate cells and background, to only measure optical redox ratio (ORR) specific to the cells. A binary mask was created by assigning values of 0 to background pixels and 1 to cell pixels. The mask was then multiplied by the raw fluorescence intensity measurements. ORR was calculated for the entire field of view as

the following: $ORR = \text{NAD(P)H} / (\text{NAD(P)H} + \text{FAD})$. ORR measurements were normalized to our baseline PEG hydrogel formulation, defined here as 3.5 mM cRGDfC and 0.5 kPa (also referred to as 3.5C 0.5).

3.3.8 Glucose / lactate assay

Cell culture media was collected on day 30 of differentiation for all samples. Glucose and lactate assays were performed on media samples using a lactate colorimetric assay kit (Biovision) and a glucose colorimetric assay kit (Biovision), as per the manufacturer's instructions. Absorbance reading was performed using a plate reader at 570 nm.

3.3.9 Ca^{2+} Intensity Measurements

On day 30 of differentiation, Ca^{2+} transient imaging was performed. Samples were washed with 1xPBS prior to incubating with 2 μM Fluo-4-AM in RPMI/B27+ media for 30 minutes at 37°C. Samples were imaged in an environmental chamber (37°C, 5% CO_2) using a Nikon TI Eclipse microscope with 4x objective with 200 ms exposure at maximum frame rate during 20 s (Supplementary Figure 3.3A). Fluorescence intensity measurements were collected from videos using NIS Elements software (Nikon). Three regions of interest (ROIs) were defined per sample as well as one background ROI (i.e. no cells present). Mean fluorescence intensity was collected for each frame at each ROI. Mean intensity measurements were normalized to background mean intensity, then normalized to minimum intensity per ROI (i.e. baseline cell fluorescence at rest) (Supplementary Figure 3.3B). Peak fluorescence intensity (F_{max} / F_0) is calculated for each sample as the average intensity of Ca^{2+} fluorescence peaks of all three ROIs and is

normalized to our baseline PEG hydrogel formulation, defined here as 3.5 mM cRGDfC and 0.5 kPa (also referred to as 3.5C 0.5).

3.3.10 MuscleMotion Video Analysis

Cells were imaged on day 30 of differentiation using a Nikon TI Eclipse microscope to acquire 20 s brightfield video recordings at 50 frames per second using a 10x objective lens. Video recordings were analyzed using MuscleMotion, an open-source software plugin compatible with ImageJ software [24]. Standard settings were used during MuscleMotion analysis, according to the manufacturer's instructions. Here, since some culture conditions support cell monolayer culture and others cell aggregate formation, the output MuscleMotion data used was solely focused on time of contraction measurements rather than contractile force measurements. Doing so helps eliminate bias in force measurements and displacement orientation caused by different numbers of cells-per-pixel during video analysis (e.g. 3D displacement for cell aggregates which cannot be captured from 2D video recordings).

3.3.11 Optimization Model

3.3.11.1 Maturation Score

To facilitate optimization modeling, the normalized results for each maturation metric (rt-qPCR, optical redox imaging, and Ca^{2+} transient imaging) were summed into one result, defined here as the maturation score. This offered equal weights for each maturation marker such that no emphasis was placed on one specific maturation marker. The maturation score was defined as follows: Maturation Score = Gene expression fold change + Ca^{2+} peak fluorescence intensity – Optical redox ratio. Since all metric values

were normalized, the maturation score has a theoretical minimum and maximum, over which we normalized the maturation score. The maturation score was calculated for each replicate. Here, to improve iPSC-CM maturation, the goal was to maximize the maturation score. A hit was defined as a PEG hydrogel formulation which supported culture of iPSC-CMs with a significantly greater maturation score than iPSC-CMs cultured on Matrigel substrate.

3.3.11.2 Bayesian Optimization Script

Our optimization model follows a Bayesian optimization (BO) process. BO is implemented using the scikit-optimize, or skopt, library in Python. Using this library, we adapted the model to work in three dimensions as our parameter space includes three hydrogel variables (primary peptide, secondary peptide, and stiffness). Note that to have a continuous axis for the primary and secondary peptides, the concentration of one peptide is negative (e.g. CRGDSP: -7 mM to 0 mM and c(RGDfC): 0 to 7 mM, CIKVAV: -3.5 mM to 0 mM and CYIGSR: 0 mM to 3.5 mM). The model was also adapted to run multiple samples per iteration since our screens contained 50 hydrogel formulations each.

3.3.11.3 Bayesian Optimization Simulations

Data simulations were used to validate the use of our BO model on our hydrogel screens. Here, we implemented a random smooth function, using the Perlin noise function from the Perlin-noise library in Python. The Perlin noise function was used as our objective function in our BO model. We applied a uniform grid search function to obtain data representing that of our first maturation screen. Using the uniform grid search function was similar to the full factorial design used to identify initial conditions in our first

maturation screen. We then applied our BO model to identify the next sample points in our Perlin noise function. We assessed the convergence of our model, over 6 consecutive screens, towards the minimum of the Perlin noise function, to identify the number of samples (and screens) necessary to identify the minimum of the function. We compared the results from these simulations to the 'true' minimum of the Perlin noise function, to assess the success of our model. The Perlin noise function was varied using different octaves (e.g. the function granularity) to account for complexity in our system. Ten distinct Perlin noise functions were generated for each octave level. To account for noise, each Perlin noise function was run through our model three times.

3.3.12 Data Analysis and Statistics

JMP statistical analysis software was used to perform multivariate analysis, by applying a standard least squares model and performing a Student's t-test. Multivariate analysis was used to assess the effect of individual and combinations of hydrogel variables on the iPSC-CM maturation score. All other statistical analyses were performed using GraphPad Prism software with Student's t-test or one-way ANOVA as indicated.

3.4 Results

3.4.1 PEG Hydrogels Offered a High Degree of Control Over Microenvironmental Properties

PEG hydrogels demonstrate substrate properties that resemble the range of ECM characteristics throughout different stages of the human heart [25]. We developed a 3-dimensional parameter space by varying the hydrogel stiffness, the primary peptide (c(RGDfC) or CRGDSP) concentration, and the secondary peptide (CYIGSR or CIKVAV)

concentration. These variables were screened in our 3-dimensional parameter space, by applying an optimization model to rapidly identify an optimized PEG hydrogel formulation (Figure 3.1). PEG hydrogel was appropriate for our screening approach as it offered a high degree of control over material properties (Figure 3.2). The PEG hydrogel stiffness was successfully varied between 0.5 kPa and 10 kPa in increments of 0.5 kPa, with a coefficient of determination of 0.9952 (Figure 3.2A). The synthetic peptides were successfully incorporated into the resulting hydrogel at concentrations up to 3.5 mM for all peptides and all hydrogel stiffnesses, with over 90 % crosslinking efficiency (Figure 3.2B). At our highest primary peptide concentration, we saw a slightly reduced crosslinking efficiency but still exceeding 75 %, and this was limited to the hydrogels with highest stiffness (Figure 3.2B). Overall, PEG hydrogels demonstrated the ability to form materials with relevant characteristics for heart development, providing the ability to screen for PEG hydrogel formulations most relevant for iPSC-CM maturation.

3.4.2 One PEG Hydrogel Formulation Improved iPSC-CM Maturation in the First Maturation Screen

By investigating three different aspects of iPSC-CM maturation, we showed that PEG hydrogels supported iPSC-CM maturation, with one formulation outperforming the standard Matrigel substrate. An initial 50 PEG hydrogel formulations were selected for screening as these conditions previously supported iPSC-CPC adhesion, survival and differentiation into iPSC-CMs [18]. For a holistic iPSC-CM maturation, we assessed structural, metabolic, and functional maturation by studying gene expressions of sarcomere protein isoforms, optical redox ratio (ORR), and Ca^{2+} handling efficiency,

respectively. At least one PEG hydrogel hit formulation, defined here as outperforming the standard Matrigel substrate, was identified for each iPSC-CM maturation marker other than the ORR (Figure 3.3A). To have a holistic view of our cells' maturation progress and to simplify our optimization model, we developed a maturation score, defined here as the following:

$$\text{Maturation Score} = \text{Gene expression fold change} + \text{Ca}^{2+} \text{ peak fluorescence intensity} + \text{ORR}$$

The iPSC-CM maturation score, defined such that it combined all three maturation markers into one unique score, varied greatly across our PEG hydrogel parameter space (Figure 3.3B, Supplementary Figure 3.4A). Overall, the maturation score for iPSC-CMs cultured on PEG hydrogels was similar to that of cells on Matrigel (Figure 3.3C). However, the formation of a long-tail behavior was observed for the maturation score of iPSC-CMs on PEG hydrogels (Figure 3.3C), indicating the possibility of PEG hydrogel formulations not tested during this first maturation screen that could maximize iPSC-CM maturation. Still, one PEG hydrogel hit formulation, our baseline formulation composed of 3.5 mM c(RGDfC) at 0.5 kPa, was identified to outperform Matrigel in terms of iPSC-CM maturation score (Figure 3.3D).

3.4.3 Optimization Modeling Identified Superior PEG Hydrogel Formulations for iPSC-Derived Cardiomyocyte Maturation

Using our BO optimization model on our hydrogel parameter space, we identified PEG hydrogel formulations able to support greater iPSC-CM maturation. Based on the results from our first maturation screen, our optimization model suggested 50 new hydrogel formulations to test in a second maturation screen (Figure 3.4A). Applying the

same process to study iPSC-CM maturation markers, we again identified at least one hit hydrogel formulation for each maturation marker, except for the ORR (Figure 3.4B). By combining these maturation markers, we observed varying iPSC-CM maturation scores depending on the hydrogel formulation (Figure 3.4C, Supplementary Figure 3.4B). The maturation score of cells cultured on screen 2 PEG hydrogel formulations remained similar to that of cells cultured on Matrigel (Figure 3.4D). However, comparing the maturation score of iPSC-CMs cultured on hydrogel formulations from screen 1 and screen 2, we observed a significant improvement in the maturation score in our second screen (Figure 3.4D). The notable increase in maturation score across both screens highlights the successful application of our optimization model to identify formulations of interest for this specific outcome. The maturation score for cells cultured on Matrigel was unchanged across the two screens (Supplementary Figure 5). Further, two hit hydrogel formulations were identified in this second maturation screen: our baseline hydrogel formulation consisting of 3.5 mM c(RGDfC) at 0.5 kPa (same as in screen 1) and one additional formulation composed of 7 mM c(RGDfC) and 3 mM CIKVAV at 2 kPa (Figure 3.4E). None of the two hit formulations identified here were shown to outperform Matrigel for any single maturation marker (Figure 3.4B), highlighting the superior holistic maturation of iPSC-CMs cultured on these two hit conditions. We also saw no difference in cTnT expression across cells cultured on Matrigel and our hit formulation (Supplementary Figure 6), emphasizing further that the optimization was targeted specifically towards iPSC-CM maturation.

All PEG hydrogel variables affected the iPSC-CM maturation score to a similar extent. Using a multivariate model, with a coefficient of determination of 0.07 (Supplementary Figure 7), we showed that none of the hydrogel variables had a significant influence on the iPSC-CM maturation score for all formulations tested across both screens 1 and 2 (Figure 3.5). The interactive effects of hydrogel variables did not have an influence on the maturation score either (Figure 3.5). This supported our hypothesis that neither the cell adhesion ligands (identity and concentration) nor the substrate stiffness has a greater influence on iPSC-CM maturation and that these variables should be manipulated simultaneously to identify optimal environmental conditions that maximize iPSC-CM maturation.

3.4.4 Additional Maturation Metrics Confirmed PEG Hydrogel Hit Formulations

Hit hydrogel formulations were validated by using the same maturation metrics to calculate iPSC-CM maturation score after seeding a different batch of iPSC-CPCs. Following the same procedure as in our maturation screens, we compared our two hit formulations to Matrigel and non-hit PEG hydrogels. Our hits outperformed Matrigel across several maturation markers (Figure 3.6A). As expected, our two hits resulted in a superior iPSC-CM maturation score, compared to Matrigel (Figure 3.6B).

Hit hydrogel conditions were further validated by assessing additional maturation markers. We confirmed the MYL2/7 gene expression results by quantifying myosin light chain (MLC2) protein expression using flow cytometry and showing greater MLC2v (mature isoform of MLC2) expression on our hit formulations compared to Matrigel (Figure 3.7A). We further demonstrated this using fluorescence imaging to show greater MLC2v

expression on our hit hydrogels compared to Matrigel (Figure 3.7B, Supplementary Figure 3.8A). Additionally, we saw an early increase in cardiac troponin I (cTnI) expression on one of our hit conditions (Supplementary Figure 3.8B). We also measured greater concentrations of glucose and lactate in media collected from cells cultured on hit hydrogels, hinting at a greater metabolic activity (Figure 3.7C). Finally, contractile measurements did not highlight specific differences in contractile speed across the different substrates (Figure 3.7D). This may indicate that the differences observed in Ca^{2+} handling may not directly translate to the cell contractility measurements studied here.

3.4.5 Modeling Simulations Identified Optimal PEG Hydrogel Formulations for Maximized iPSC-Derived Cardiomyocyte Maturation Within Two Screens

Our optimization model identified the maximum of our parameter space within two screens using simulated data. To assess the number of screens (or conditions sampled) necessary to identify the maximum of our parameter space, we tested our optimization model on multiple Perlin noise functions (Figure 3.8A). Knowing the input function and therefore its true maximum, we assessed the convergence towards this maximum using our BO model to sample points within our parameter space (Figure 3.8B). The use of BO, instead of a grid sampling approach, resulted in a reduced relative difference between the true and measured maximum of our parameter space, with a significant decrease in the case of lower octaves, i.e. less complex parameter space (Figure 3.8C). Overall, regardless of the complexity of our function, our simulated results indicate that the maximum is identified within 2 screens on average (Figure 3.8D). By performing 2 screens, we identified the simulated parameter space's maximum with greater than 85 %

accuracy, across all octave levels tested here (Figure 3.8E). By performing 2 maturation screens here, the optimal PEG hydrogel formulations to maximize iPSC-CM maturation, within our parameter space, are the 3.5 mM c(RGDfC) at 0.5 kPa and 7 mM c(RGDfC) 3 mM CIKVAV at 2 kPa formulations, with greater than 85 % confidence.

3.5 Discussion

PEG hydrogel synthesis offered high tunability of certain material variables, supporting screening of a large parameter space. Varying both hydrogel shear modulus and adhesion ligand type and concentration, we developed PEG hydrogels demonstrating precise control over material properties (Figure 3.2), throughout the range relevant for iPSC-CPC and -CM culture [25,26]. Matrigel lacks control over these material variables and can be unpredictable across batches [17]. PEG-based materials are generally a valuable synthetic alternative for their tunability and ease of synthesis [27]. Use of thiol-ene based polymerization specifically supported the crosslinking of a variety of different molecules, at a variety of concentrations [28,29]. Here, we identified two hit PEG hydrogel formulations, both demonstrating low shear moduli and cRGDfC peptides. Other studies focusing on single material variables appeared to show similar results. Softer substrates have demonstrated greater CM maturation, as shown by increased myofibril alignment, mature gene expression, and more adult-like contractility [25,30-33]. Fibronectin and fibronectin-derived products have also been shown to play a crucial role in iPSC-CM survival [34,35]. Here, we showed that these conclusions hold when varying both hydrogel stiffness and adhesion ligand identity and concentration. We also showed that the “one size fits all” approach, generally applied in cell culture by using Matrigel

regardless of cell type or desired outcome, is not the ideal approach and that cell culture could benefit from substrates tailored to a specific application.

The screening approach developed here identified two hit PEG hydrogel formulations, significantly outperforming iPSC-CM maturation levels observed on Matrigel. We compared our platform here to Matrigel as it is the standard stem cell culture and cardiomyocyte differentiation platform [17,19]. In doing so, we showed that iPSC-CM maturation could be furthered by using our hit PEG hydrogel formulations as an alternative platform (Figure 3.4), even on a short maturation timeline of 30 days here. Other studies using different approaches have shown significant improvements in iPSC-CM maturation, with superior gene and protein expression [6,36], metabolic maturation [37,38], and mechanical output [6,13,39,40]. We expect these studies to yield greater CM maturation than observed here, as we demonstrated only minor improvements. However, many of the techniques used in these studies can be labor intensive and require highly trained workers or specific instruments. Here, we developed an alternative material to the standard Matrigel substrate that is essentially as simple to generate and could easily be substituted into the iPSC-CM manufacturing workflow.

Our multi-screen optimization process supported rapid identification of regions of interest within our parameter space, quickly leading to hit discovery. Screening can be limited by sample size, material fabrication technique, or lengthy experimental setups [28]. Combining a screening approach with an optimization model can significantly improve screening efficiency. An increasing number of studies are applying modeling tools to optimize cell differentiation processes [41-43]. Williams et al. developed a

multifactorial process modeling tool to identify experimental conditions, such as pH levels and nutrient availability, necessary for hPSC-CM differentiation [44]. Studies looking at cell maturation may greatly benefit from the use of modeling tools, as maturation remains broadly defined, requiring multiple experimental readouts. Here, we showed the successful application of an optimization model to identify material formulations specific to iPSC-CM maturation. The use of this optimization model could be generalized to screening applications for other cell behavior of interest.

3.6 Conclusion

The cell's microenvironment plays a key role in determining cell fate. Successful iPSC-CM manufacturing is dependent on the correct microenvironment. Here, we identified two specific PEG hydrogel formulations that support high iPSC-CM maturation. These two substrates were identified by using a multi-parametric, multi-screen approach combined with optimization modeling to efficiently assess a large number of possible substrate formulations. The successful application of an optimization model here highlights the broad impact that optimization modeling can have on cell culture and manufacturing applications.

3.7 Acknowledgements

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3.8 References

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3.9 Figures

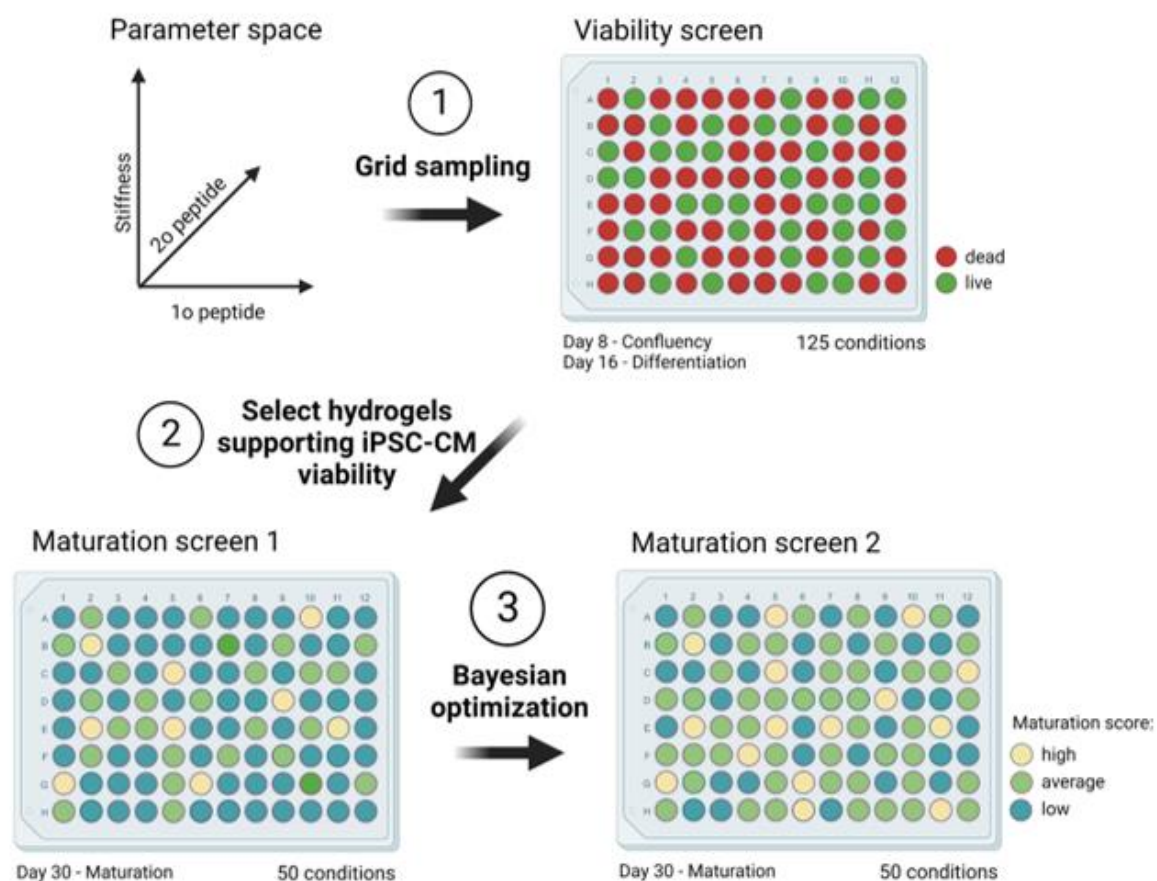


Figure 3.1. Screening workflow used to identify PEG hydrogel formulations that support iPSC-CM maturation.

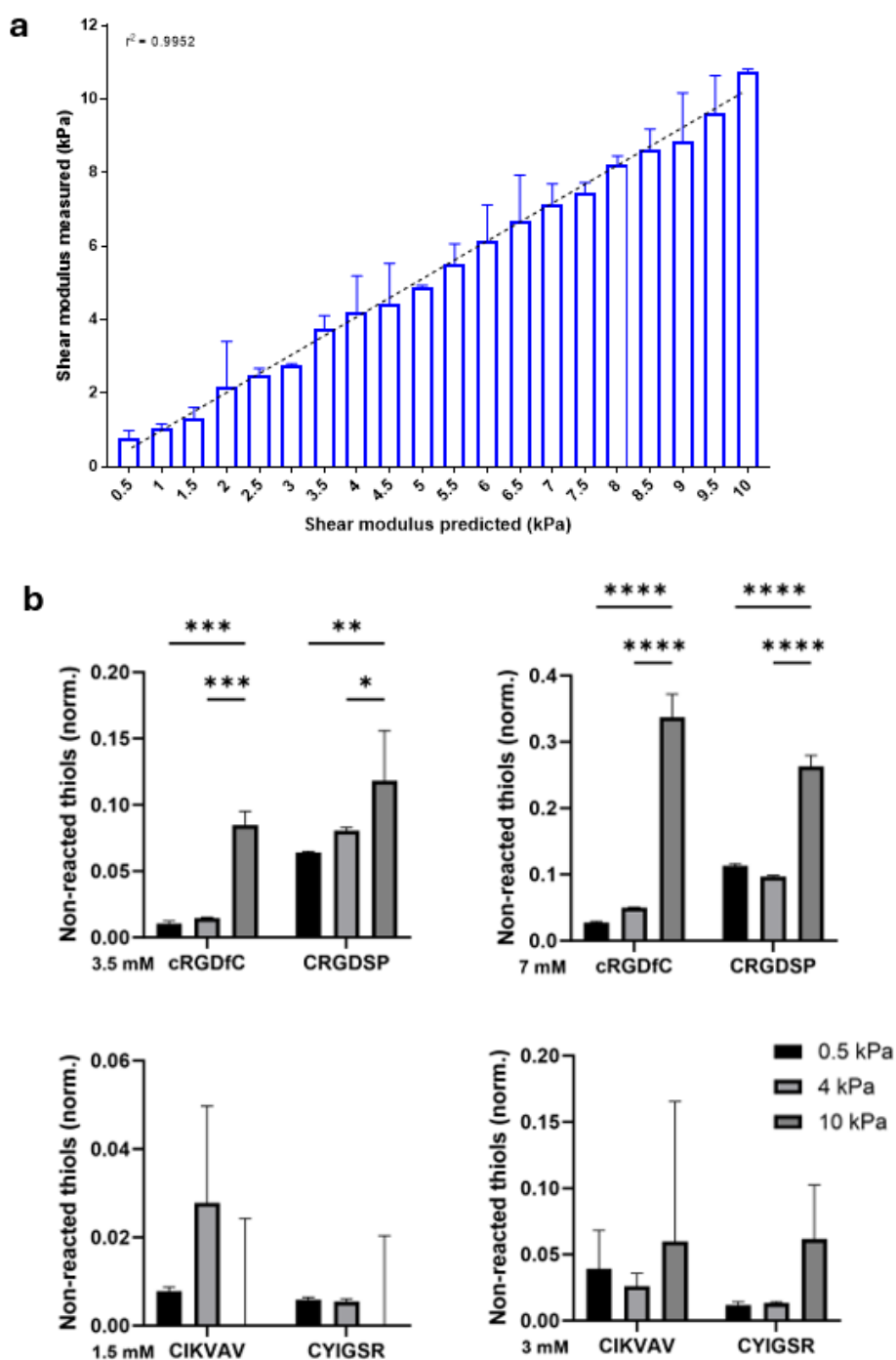


Figure 3.2. PEG hydrogels offered precise control over material properties.

(A) Rheology measurements of PEG hydrogel shear modulus. **(B)** Non-reacted thiol measurements of varying peptide type and concentration after PEG hydrogel polymerization.

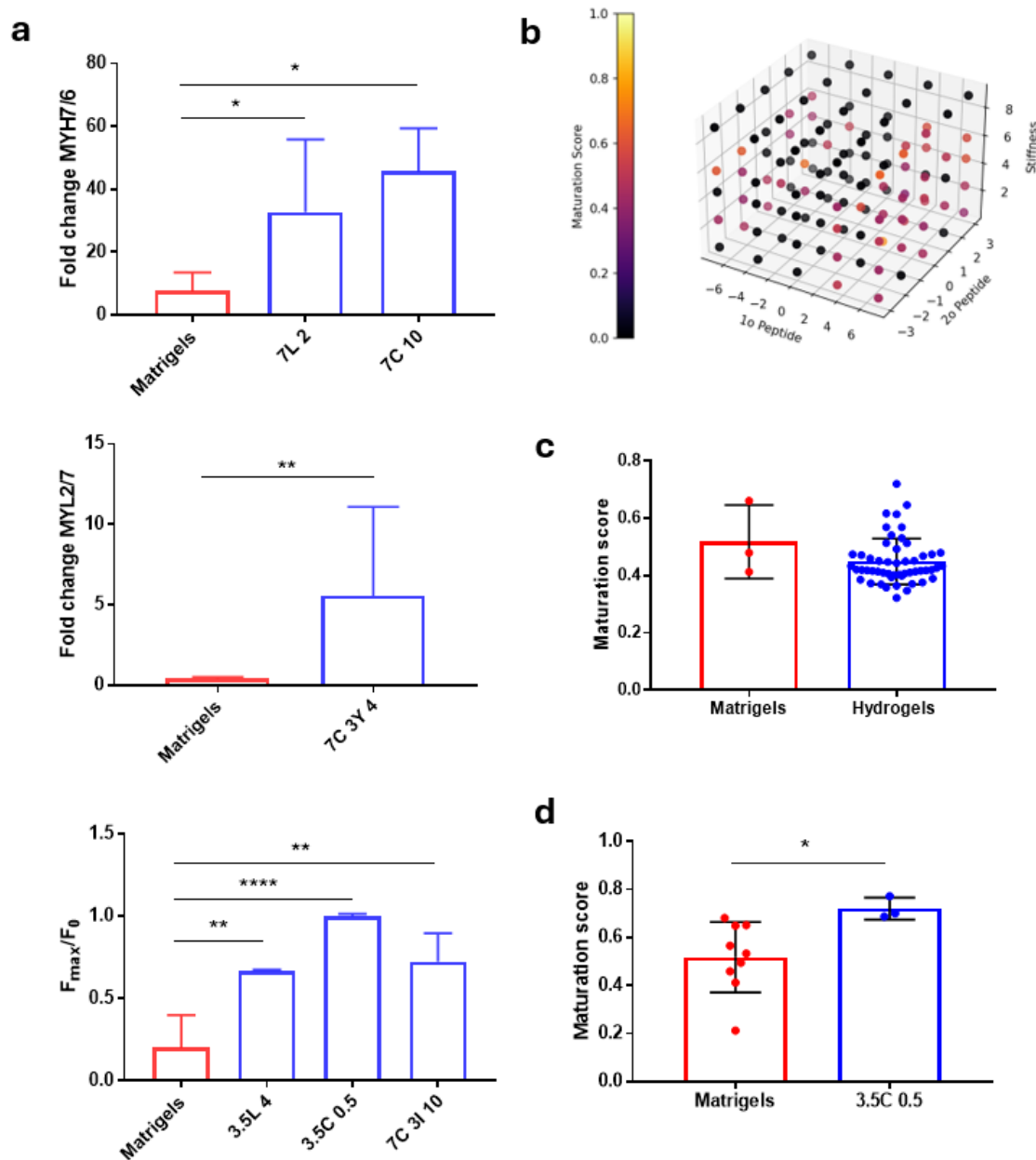


Figure 3.3 PEG hydrogels supported maturation of iPSC-CMs.

(A) Comparison of hit hydrogel formulations to Matrigel for the maturation metrics evaluated. * $p < 0.05$, ** $p < 0.01$, one-way ANOVA. **(B)** 3D plot representative of iPSC-CM maturation score in varying PEG hydrogel conditions. **(C)** Comparison of iPSC-CM maturation scores on Matrigel and PEG hydrogels. Note that one dot represents three replicates. ns: no statistical significance, Student t-test. **(D)** Maturation score of iPSC-CMs cultured on Matrigel or hit hydrogel. Note that one dot represents one replicate. * $p < 0.05$, Student t-test.

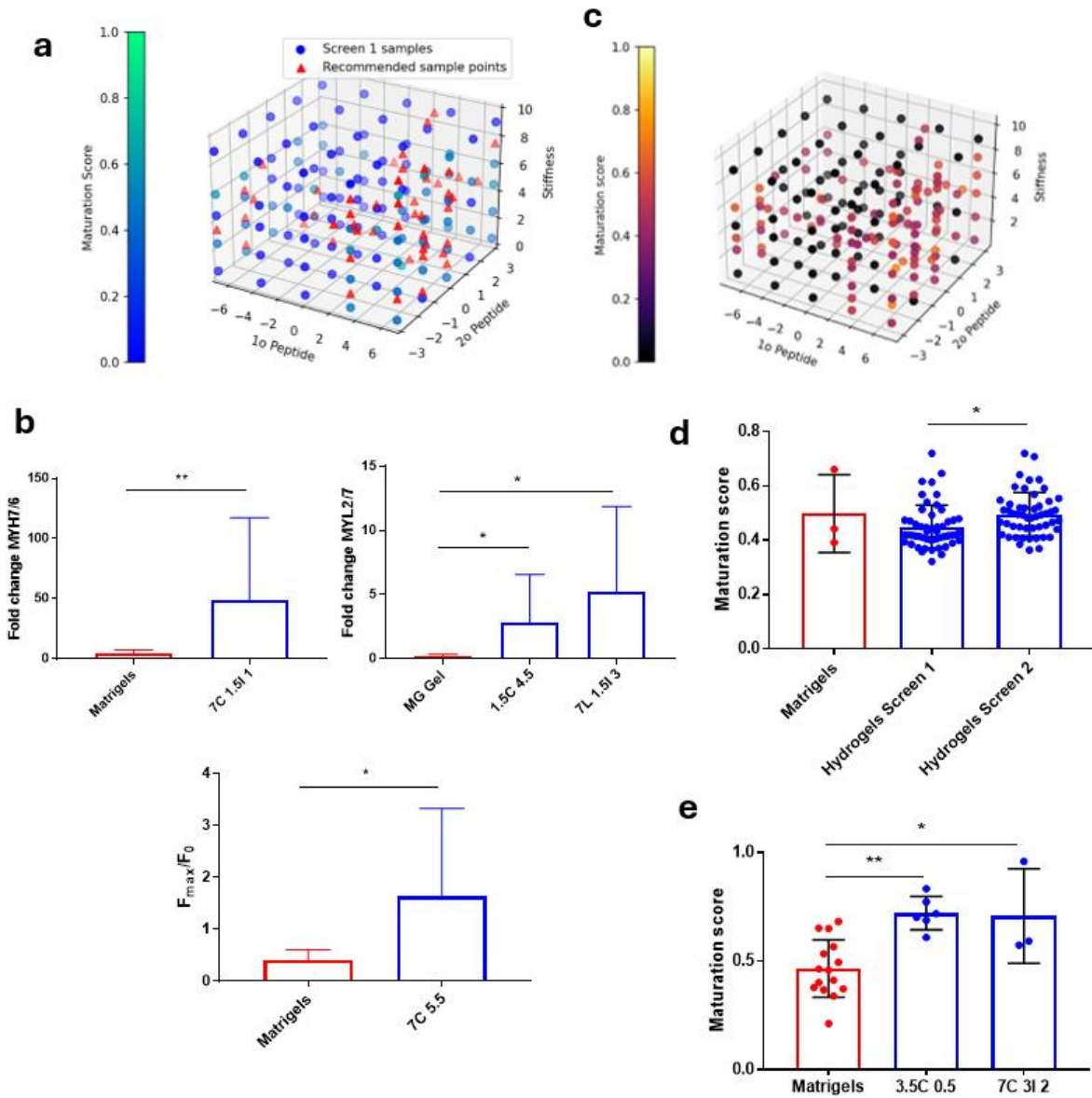


Figure 3.4 Screening of PEG hydrogels identified hit formulations that support robust iPSC-CM maturation.

(A) 3D plot of suggested hydrogel formulations using BO modeling. **(B)** Comparison of hit hydrogel formulations to Matrigel for each maturation metric evaluated. * $p < 0.05$, ** $p < 0.01$, Student t-test. **(C)** 3D plot representing maturation score for all PEG hydrogel conditions tested. **(D)** Comparison of maturation score for PEG hydrogel conditions tested across different screens. ** $p < 0.01$, Student t-test. **(E)** Comparison of maturation score for iPSC-CMs cultured on Matrigel and hit hydrogel formulations. * $p < 0.05$, ** $p < 0.01$, one-way ANOVA.

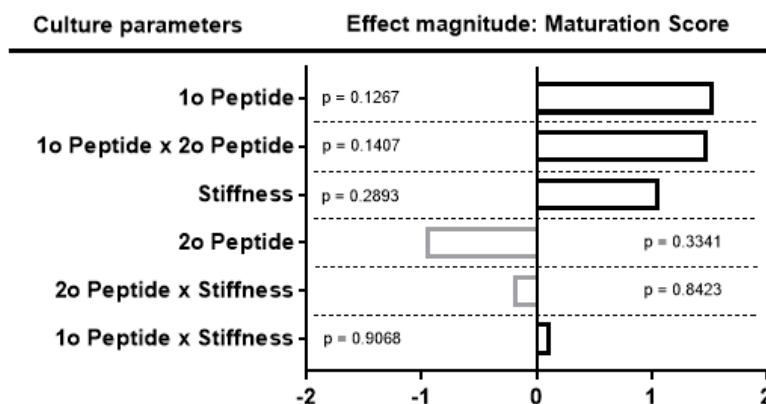


Figure 3.5. Multivariate analysis of main and interactive effects of PEG hydrogel variables on the maturation score of iPSC-CMs.

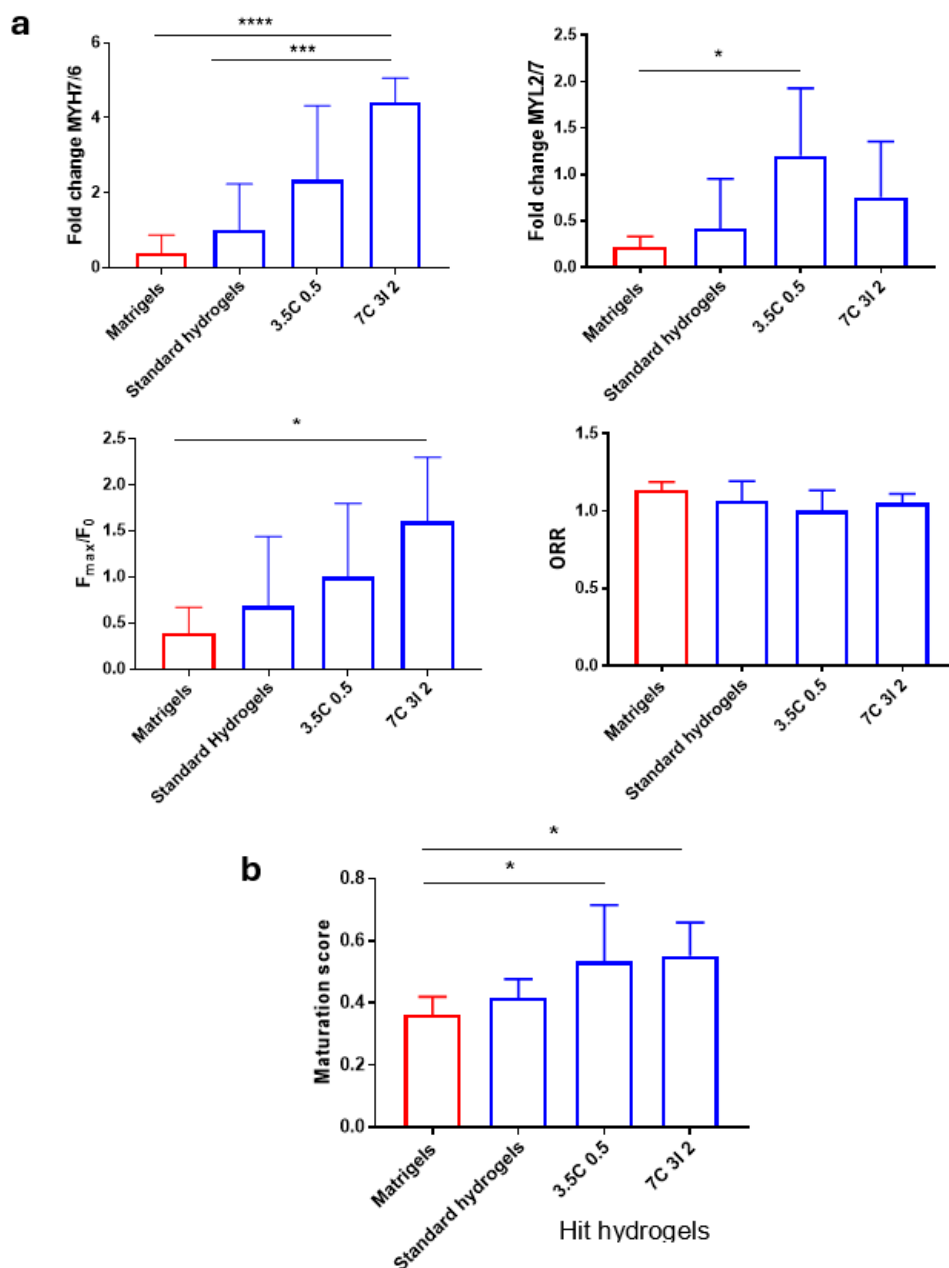


Figure 3.6. Hit PEG hydrogel formulations confirmed via repeat experiment.

PEG hydrogel hit formulations were confirmed via the same maturation metrics as the maturation screens, by **(A)** Comparison of hit conditions with Matrigel and non-hit standard hydrogel conditions, **(B)** Comparison of iPSC-CM maturation score across different materials. * $p < 0.05$ *** $p < 0.001$ **** $p < 0.0001$, one-way ANOVA.

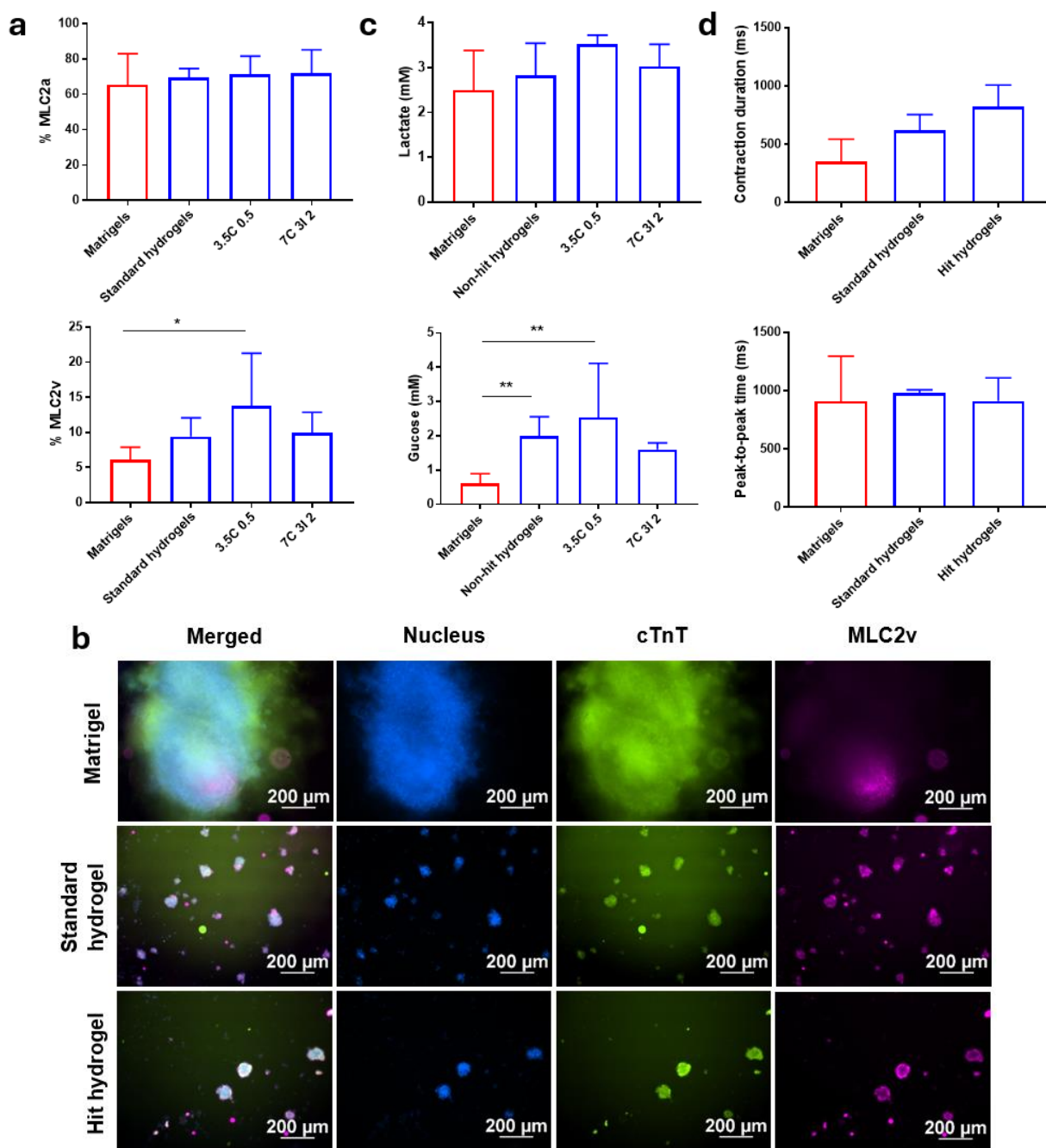


Figure 3.7 Hit PEG hydrogel formulations confirmed via additional metrics.

PEG hydrogel hit formulations were confirmed via additional maturation metrics, including **(A)** flow cytometry to quantify sarcomere protein expression, **(B)** fluorescence imaging of sarcomere proteins, **(C)** glucose and lactate assays to study cell metabolic activity, and **(D)** MuscleMotion video analysis to analyze cell contractility. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$, one-way ANOVA.

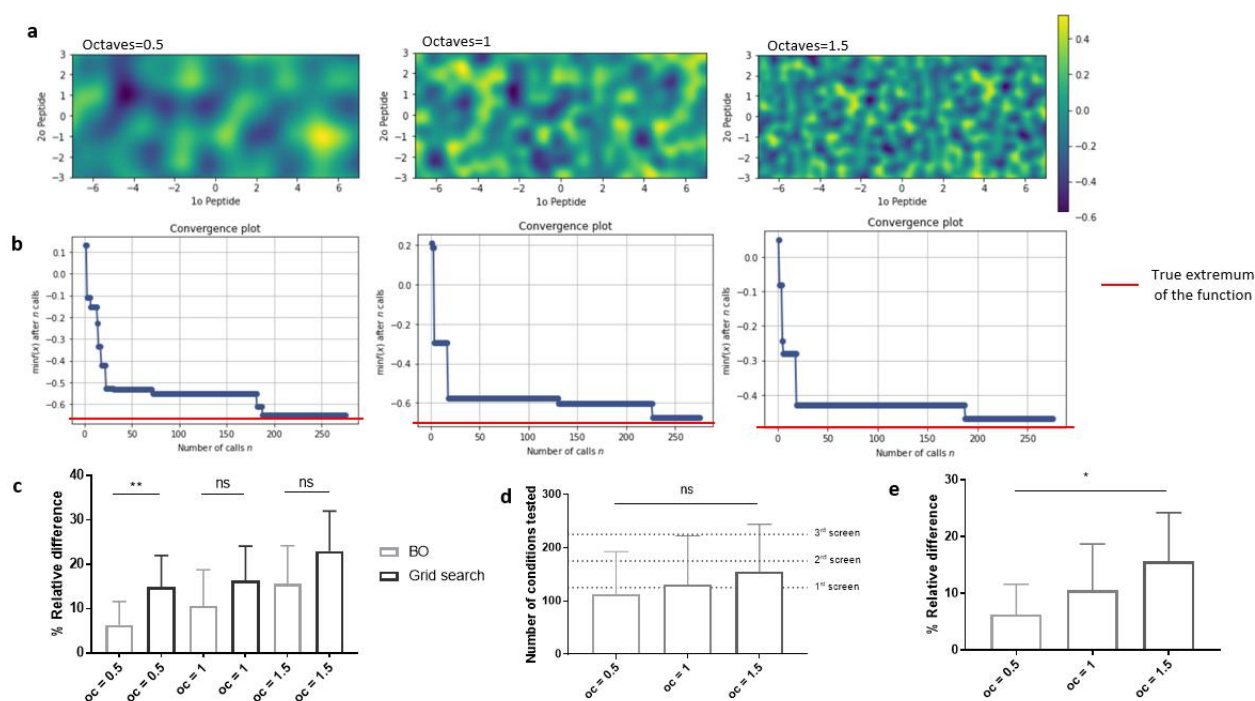
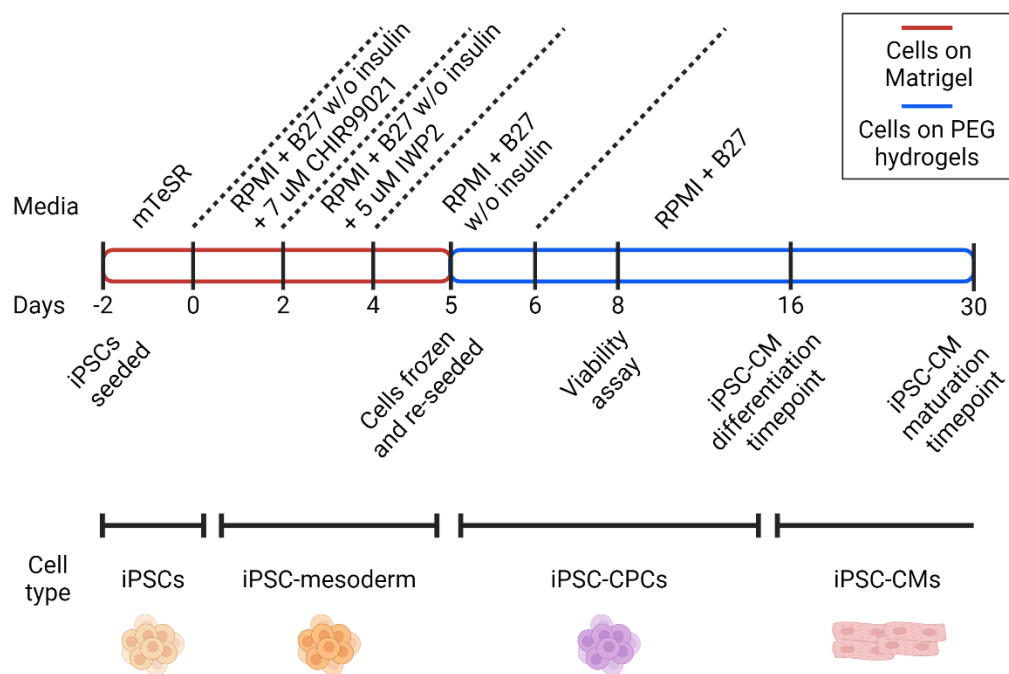
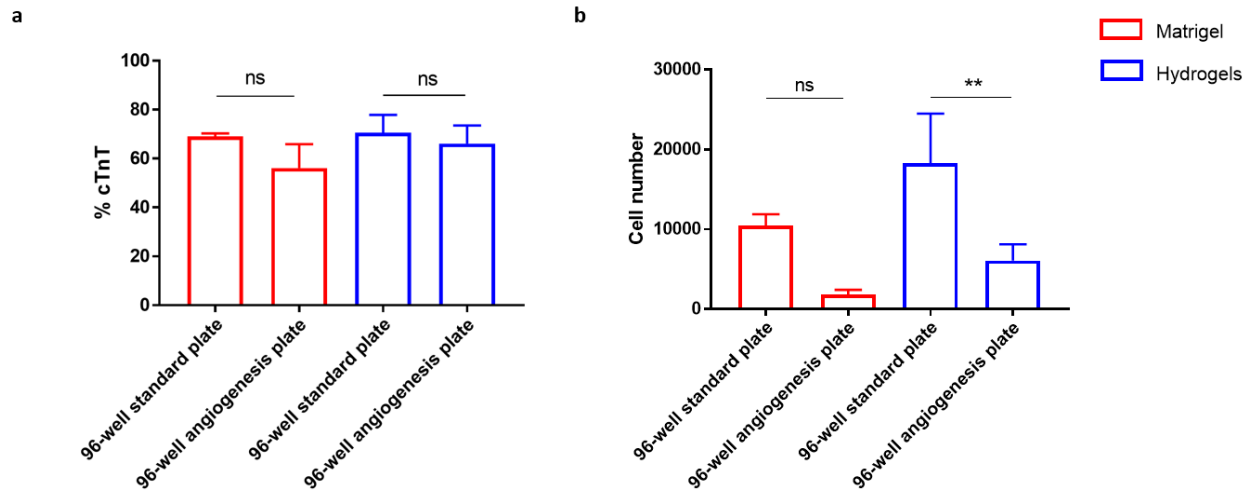


Figure 3.8. Bayesian optimization (BO) modeling supported the identification of an optimal PEG hydrogel formulation for iPSC-CM maturation within 2 screens. (A) 2D representation of a slice of our parameter space for functions of varying octaves. (B) Visual representation of the convergence towards a function's extremum using BO. (C) Relative difference between a function's true extremum and its measured extremum using different sampling techniques. Each bar represents the average result for 10 different Perlin noise functions. oc = octave. * $p < 0.05$, ** $p < 0.01$, Student t-test. (D) Quantification of the number of hydrogel conditions tested to identify an extremum for functions with different octave levels. (E) Relative difference between a function's actual and measured extremum for varying octave levels.

3.10 Supplemental Figures

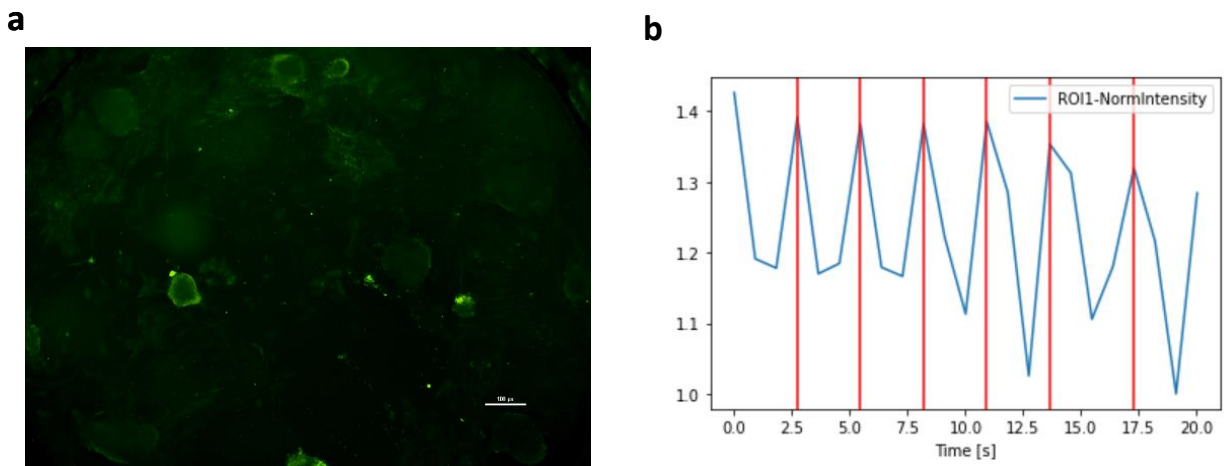


Supplementary Figure 3.1. Schematic representation of the iPSC-CM differentiation and maturation timeline used throughout this study.



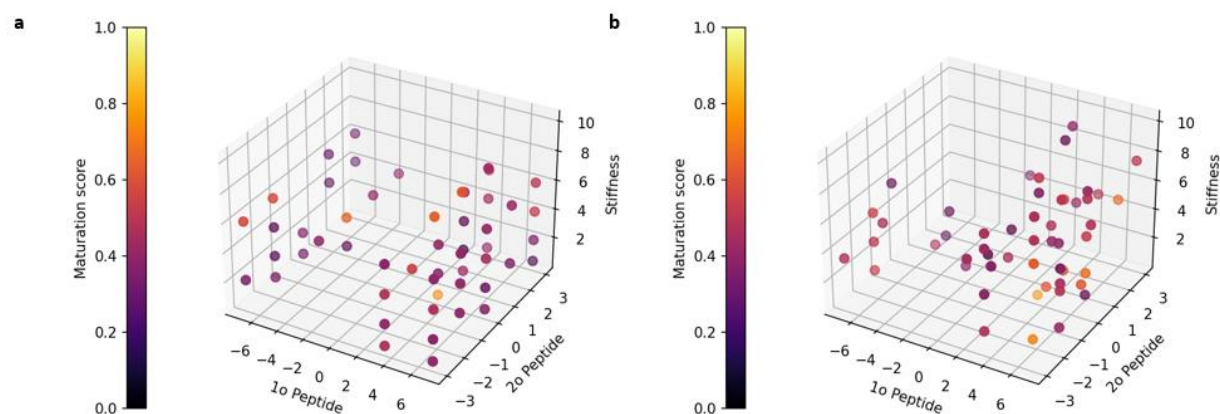
Supplementary Figure 3.2. Differences in iPSC-CM differentiation and yield across different plate sizes.

Flow cytometry quantification of **(A)** % cTnT-expressing cells, and **(B)** total cell number, on Matrigel and PEG hydrogels in plates of different sizes. ns: no statistical significance
 ** $p < 0.01$, Student t-test.



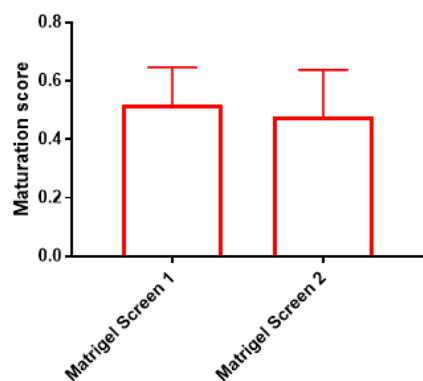
Supplementary Figure 3.3. Processing of Ca^{2+} fluorescence recordings.

(A) Fluorescent video recording of iPSC-CMs, stained with Fluo-4-AM, cultured on PEG hydrogel. **(B)** Fluorescence intensity over time, normalized to background intensity, extracted from fluorescent video recording.

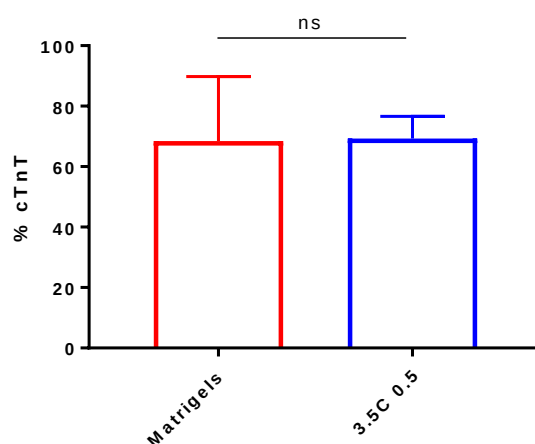


Supplementary Figure 3.4. Maturation score of conditions tested.

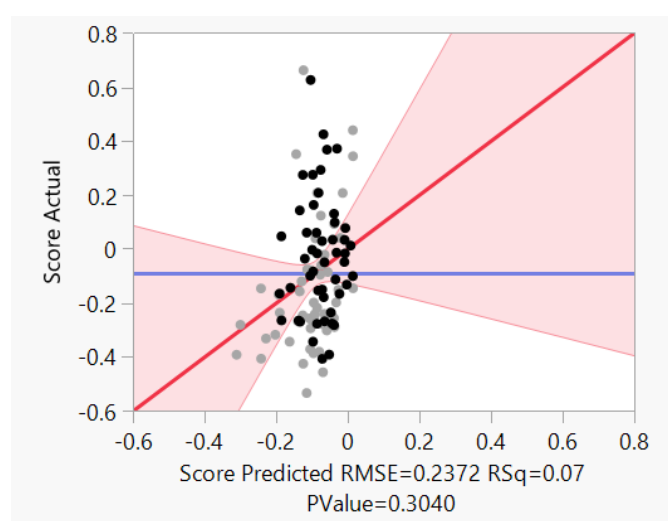
3D plot representing maturation score for all PEG hydrogel conditions tested in **(A)** maturation screen 1, and **(B)** maturation screen 2.



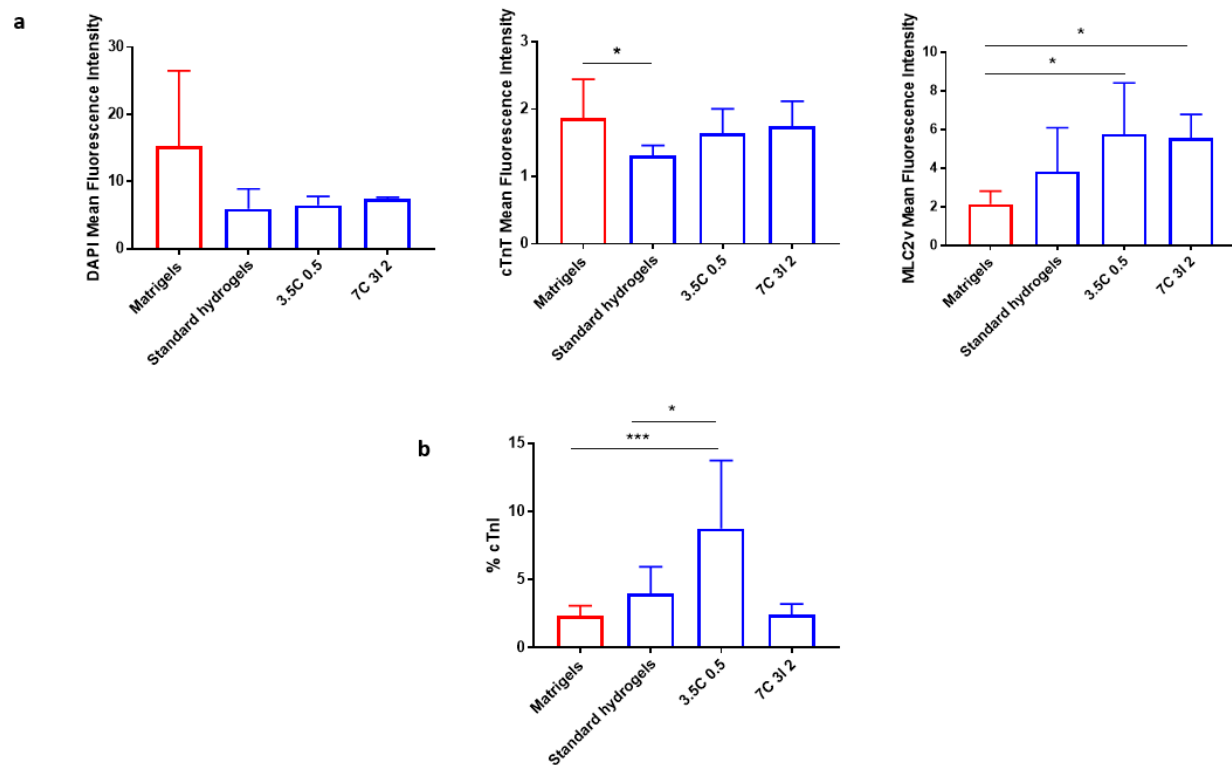
Supplementary Figure 3.5. Comparison of maturation score for iPSC-CMs cultured on Matrigel substrate in maturation screens 1 and 2.



Supplementary Figure 3.6. Comparison of % cTnT expression on Matrigel and our hit PEG hydrogel formulation. ns: no statistical significance, Student t-test.



Supplementary Figure 3.7. Actual by predicted plots of standard least squares models of the effect of hydrogel variables on the maturation score.



Supplementary Figure 3.8. PEG hydrogel hit formulations were further confirmed with additional measurements.

(A) Fluorescence intensity measurements extracted from fluorescence images (Figure 3.6D). **(B)** Flow cytometry quantification of cardiac troponin I (cTnI) expression. * $p < 0.05$
*** $p < 0.001$, one-way ANOVA.

Chapter 4 : Effect of PEG Hydrogel Surface Topography on iPSC-Derived Cardiomyocytes

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4.1 Abstract

Stem cell-derived cardiomyocytes (iPSC-CMs) have a high therapeutic potential that remains unmet due to their limited maturation. Adult cardiomyocytes demonstrate high cell and tissue level organization that contributes to their robust functional output. The cell's physical microenvironment plays a crucial role in cell development. Here, PEG hydrogels were manipulated to vary surface topography such that iPSC-CMs were cultured on micro-patterned grooves of 4 μm depth and 50 – 100 μm width. Surface topography resulted in short-term cell and aggregate alignment along the defined pattern. However, this short-term alignment resulted in long-term myofibril and nuclear alignment in iPSC-CMs. Surface topography had a limited influence on iPSC-CM protein expression. These results highlight the importance of the cell culture substrate, specifically the surface micro-scale topography, and its role in iPSC-CM maturation.

4.2 Introduction

Stem cells have a high therapeutic potential as their population doubling time is quite short and they have the ability to differentiate into many different cell types. Stem cell differentiation into specific lineages is highly dependent on the cells' microenvironment, including cell-material interactions. Induced pluripotent stem cells (iPSCs) can successfully be differentiated into cardiomyocytes (CMs) [1-3]. The effect of soluble factors on stem cell-derived cardiomyocyte (iPSC-CM) differentiation is largely established especially as soluble factors are the main factor used to direct stem cell differentiation towards a CM lineage [2]. Soluble factors are also extensively studied in CM maturation, with various combinations identified to further CM development into more mature phenotype [4-8]. The knowledge of the effect of insoluble factors on iPSC-CM differentiation and maturation remains more limited, especially considering that the cardiac microenvironment is highly dynamic during development and disease.

Many iPSC-CM differentiation protocols generate cells with limited maturation as they largely resemble embryonic or fetal CM phenotypes [9-11]. These cells lack a number of properties found in adult CMs, which greatly limit their applicability in drug screening and modeling adult diseases. A major difference in phenotypic development of iPSC-CMs, in comparison with adult CMs, is their lack of organization, both at the cell and tissue level. Adult CMs demonstrate anisotropic, rod-like morphologies, whereas iPSC-CMs remain rounded during culture [9-11]. The high aspect ratio of adult CMs contributes to their intracellular organization through greater myofibril alignment which in turn results in more robust mechanical output [9]. Adult CMs also organize with their

neighboring cells along a specific orientation at the intercellular level. This supports gap junction polarization at the intercalated discs which results in more robust electrical conduction throughout the tissue [9,12-15]. iPSC-CMs, however, are greatly limited in their intracellular and intercellular organization, resulting in weak electrical and mechanical output at the cell and tissue level [9,14,15].

We had previously looked at optimization of the substrate stiffness and adhesion ligand type and concentration for iPSC-CM differentiation and maturation [16]. From this, two hit PEG hydrogel formulations were identified: 3.5 mM c(RGDfC) at 0.5 kPa and 7 mM c(RGDfC) 3 mM CIKVAV at 2 kPa. These two PEG hydrogels supported high levels of iPSC-CM maturation [16]. Here, we propose to build on this study and to study the effect of hit PEG hydrogel surface topography on iPSC-CM maturation by directing the cells to align and organize along a defined pattern. We hypothesized that by guiding iPSC-CM organization on hit PEG hydrogels, iPSC-CM maturation may be further improved and iPSC-CMs may demonstrate a more adult-like phenotype.

4.3 Methods

4.3.1 iPSC Culture and Maintenance

WTC11 iPSCs were cultured on plates coated with Matrigel (Corning) at 37°C and 5% CO₂ and fed with mTeSR medium (Stem Cell Technologies). Daily media changes were performed and cells were passaged every 3-4 days when cell confluency reached 70-90 % onto freshly coated Matrigel plates at a ratio of 1-to-12 wells. Cells were passaged by incubating in Versene (Life Technologies) prior to resuspending in mTeSR medium and 5 µM Y-27632 dihydrochloride (R&D Systems).

4.3.2 iPSC-Derived Cardiomyocyte Differentiation

iPSCs were differentiated to cardiomyocytes (iPSC-CMs) as previously described [2]. Briefly, cells were passaged onto freshly-coated Matrigel plates at a density of 40,000 cells/cm² in mTeSR medium with 5 μ M Y-27632 dihydrochloride (R&D Systems). iPSCs were cultured for 2 days in mTeSR medium such that they expanded to 80-90 % confluency. To induce differentiation, the culture medium was changed to RPMI1640 (Thermo Fisher Scientific) supplemented with B27 minus insulin (Thermo Fisher Scientific) and 7 μ M CHIR99021 (Selleck Chemicals). After 48 hours, the culture medium was replaced with RPMI1640 supplemented with B27 minus insulin and 5 μ M IWP2 (Tocris). After an additional 48 hours, cells were fed with fresh RPMI1640 supplemented with B27 minus insulin. Here, iPSC-derived cardiac progenitor cells (iPSC-CPCs; day 5 of differentiation) were singularized using Accutase (Sigma Aldrich) and transferred to freezing solution made up of 60% RPMI1640 medium with B27 minus insulin supplement, 30% FBS (Gibco), and 10% DMSO (Fisher Scientific). The frozen cell solution was stored in liquid nitrogen. iPSC-CPCs were then re-plated on Matrigel or PEG hydrogel in RPMI1640 medium with B27 minus insulin supplement and 5 μ M Y-27632 dihydrochloride. After 24 hours, cell culture medium was replaced with fresh RPMI1640 supplemented with B27 supplement (Thermo Fisher Scientific). Cells were cultured in this medium with media changes every 2-3 days for the remainder of the differentiation and maturation.

4.3.3 PEG Hydrogel Synthesis

4.3.3.1 PEG Hydrogel Manufacturing

Poly(ethylene glycol) (PEG) hydrogels were developed as previously described [16-19]. Briefly, the hydrogel precursor solution was developed by combining 8-arm, 20 kDa norbornene-functionalized PEG (PEG-NB; Jenkem Technology) with 3.4 kDa PEG di-thiol (PEG-DT; Laysan Bio) and 0.1 wt/wt % Irgacure 2959 (CIBA). To reach desired PEG-NB and PEG-DT concentrations, the solution was diluted with DPBS (Gibco). The hydrogel formulations developed here were identified as hit hydrogel conditions for iPSC-CM differentiation and maturation in a previous study [16]. Cell adhesion ligands were incorporated into the hydrogel precursor solution by adding synthetic peptides (GenScript). Here, we specifically added head-to-tail cyclized RGDfC (cRGDfC) and CIKVAV at a concentration of either 3.5 mM or 7 mM and 3 mM, respectively. Hydrogel stiffness, measured as the shear modulus, was manipulated by varying the concentration of PEG-NB and PEG-DT. Here, we varied the shear modulus within a physiologically relevant range for cardiac development [20-23]. The hydrogel precursor solution was exposed to ultraviolet light (UV, 365 nm) at 4.5 mW/cm² for 5 minutes for polymerization. The resulting hydrogels were incubated in RPMI1640 medium at 37°C until use.

4.3.3.2 PEG Hydrogel Surface Patterning

To manipulate the surface of our PEG hydrogels, photolithography was applied to etch the desired photomask (FineLine Imaging) pattern onto a silicon wafer. Molds were milled according to the dimensions of 3 wells of a 96-well angiogenesis plate (Ibidi) and taped onto the silicon wafer. Polydimethylsiloxane (PDMS) solution was developed by

mixing the base monomer with a curing agent at a 10-to-1 ratio (Fisher Scientific) and incubating in a dessicator at room temperature for an hour. The PDMS solution was poured into the molds on our silicon wafers and placed to cure on a heating platform at 90°C for 30 minutes. The PDMS stamp was then used to pattern the surface of our PEG hydrogels by applying it to the surface of the hydrogel precursor solution in a 96-well angiogenesis plate throughout UV exposure.

4.3.3.3 PEG Hydrogel Characterization

The hydrogel stiffness was measured here as shear modulus via rheological measurements, as previously described [17,18]. Briefly, PEG hydrogels of 8.0 mm diameter and 1.2 mm height were developed and incubated in PBS at 37°C until testing. Measurements were collected on the Ares-LS2 rheometer (TA Instruments) by placing the hydrogel sample between parallel plates of the same dimensions and applying a 20 g force. A dynamic strain sweep test with 1 Hz frequency was performed between 0.1 and 50 % strain and the shear modulus was measured between 1 and 10 % strain.

Peptide crosslinking into the hydrogel was measured as previously described [17]. Briefly, the remaining non-reacted thiol content in solution was measured after hydrogel polymerization using the Ellman's assay (Thermo Fisher Scientific). Hydrogels were incubated in Ellman's buffer for 20 minutes prior to absorbance reading at 412 nm on a plate reader. A standard curve using L-Cysteine hydrochloride was used to measure remaining thiol content in the hydrogel.

The surface topography of PEG hydrogels and Matrigel was imaged using a 3D optical profilometer (Zygo 9000). Samples were prepared in 96-well plates and incubated

in PBS for at least 24 hours prior to imaging. Excess PBS was removed immediately prior to imaging. The depth and width of patterned grooves were measured.

4.3.4 Flow Cytometry

Protein expression was assessed using flow cytometry for cells after 25 days in culture as previously described [2]. Briefly, cells were singularized using Accutase and transferred to DMEM/F12 medium (Thermo Fisher Scientific). Samples were centrifuged at 200xg for 5 minutes and the cells were resuspended in 1 % formaldehyde solution and incubated in the dark for 20 minutes at room temperature. Cells were centrifuged and incubated in 90 % methanol solution in the dark for 20 minutes at 4°C. Samples were then placed at -20°C until use. After washing the samples twice in Flow Buffer 1 (PBS containing 0.5 % BSA), cells were incubated in Flow Buffer 2 (PBS containing 0.5 % BSA and 0.1 % Triton X-100) with primary antibodies overnight at 4°C. After another washing step, cells were incubated in Flow Buffer 2 with secondary antibodies for 30 minutes at room temperature in the dark. The cells were resuspended in Flow Buffer 1 and placed at 4°C until processing. Data was collected using an Attune flow cytometer (Thermo Fisher Scientific). Data analysis was conducted using FCS Express software. Primary antibodies used were mouse anti-human cTnT (Abcam, 1:800), rabbit anti-human MLC2v (ProteinTech, 1:200), rabbit anti-human RYR2 (Abcam, 1:100), and goat anti-human Connexin-43 (Abcam, 1:100). The secondary antibodies used were donkey anti-mouse AlexaFluor 488 (Thermo Fisher Scientific, 1:1000), donkey anti-rabbit AlexaFluor 568 (Thermo Fisher Scientific, 1:1000), and donkey anti-goat AlexaFluor 647 (Thermo Fisher Scientific, 1:1000).

4.3.5 Imaging

4.3.5.1 Live Cell Imaging

Live cell imaging was performed using a TI Eclipse microscope (Nikon) equipped with an incubation chamber for live/dead imaging assays and timelapse imaging. The incubation chamber was set to 37°C, 5 % CO₂. For live/dead assays, the cell culture medium was changed to RPMI1640 with B27 supplement and 1.5 µM ethidium homodimer-1 and 1 µM calcein-AM (Thermo Fisher Scientific). Samples were incubated at 37°C for 30 minutes. After imaging, samples were washed with PBS before adding fresh RPMI1640 medium with B27 supplement. For timelapse imaging of the initial 24 hours in culture, cells were seeded onto the culture substrate in RPMI1640 medium with B27 minus insulin supplement and 5 µM Y-27632 dihydrochloride and placed in the incubation chamber for the duration of the imaging timelapse. Brightfield images were collected every 30 minutes over a 24-hour period. For timelapse imaging of the 48-72 hours in culture, cells were first stained with 2 µM Hoechst 33342 (Thermo Fisher Scientific). Fluorescent images were collected through the DAPI filter (460/50 nm) every 30 minutes over a 24-hour period. Finally, z-stack images were collected for cells stained with 2 µM Hoechst 33342 and 5 µM CellTracker Red (Thermo Fisher Scientific) after 24 hours in culture.

4.3.5.2 Immunocytochemistry

Imaging was performed using an AXR confocal microscope (Nikon), on cells fixed at various culture timepoints (25 days in culture if not specified). Cells were fixed on the culture substrate by incubating in 4 % formaldehyde for 20 minutes in the dark. Samples

were stored in PBS at 4°C until staining. Samples were incubated in Flow Buffer 2 for 1 hour at room temperature prior to incubating in Flow Buffer 2 with primary antibodies at 4°C overnight. Three washing steps were performed with PBS for 10 minutes each. Samples were then incubated in Flow Buffer 2 with secondary antibodies for 1 hour in the dark at room temperature. After washing an additional three times for 10 minutes each, fresh PBS was added, and samples were placed at 4°C until imaging. The primary antibodies used were mouse anti-human cTnT (Abcam, 1:200), rabbit anti-human MLC2v (ProteinTech; 1:200), goat anti-human Connexin-43 (Abcam, 1:100), rabbit anti-human Vinculin (Abcam, 1:400), and goat anti-human NKX2.5 (Abcam, 1:100). The secondary antibodies used were donkey anti-mouse AlexaFluor 488 (Thermo Fisher Scientific, 1:1000), donkey anti-rabbit AlexaFluor 568 (Thermo Fisher Scientific, 1:1000), and donkey anti-goat AlexaFluor 647 (Thermo Fisher Scientific, 1:1000). DAPI was used for nuclear staining (Thermo Fisher Scientific, 1:1000).

4.3.6 Data Analysis and Statistics

ImageJ software was used with the Orientation J plugin to measure orientation angle distribution of cells and to generate colorized maps of cell orientation [24]. A Lorentzian distribution was used to fit the angle distribution data at each timepoint. From these fitted curves, the full width at half maximum (FWHM) and the angle of the center of peak were analyzed using OriginLab software. Additional statistical analysis was performed using GraphPad Prism software using Student t-test, one-way or two-way ANOVA. The statistical significance was considered as follows: ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

4.4 Results

4.4.1 PEG Hydrogel Surface Topography Was Successfully Manipulated With a High Degree of Control Via a Patterning Approach

PEG hydrogels were highly amenable to modifications, including their surface topography (Figure 4.1). To manipulate the surface of PEG hydrogels, PDMS stamps demonstrating the desired pattern were developed from a micro-patterned mold developed via photolithography. These PDMS stamps were applied to the surface of the hydrogel precursor solution prior to photo-crosslinking such that the desired pattern was transferred to the surface of the PEG hydrogel during polymerization (Figure 4.1). The surface of our materials was characterized by optical profilometry, which showed clearly defined, parallel grooves on the surface of the PDMS stamp and the PEG hydrogels (Figure 4.2A). By varying the groove dimensions, we demonstrated precise control over the surface topography profile of our PDMS stamps (Supplementary Figure 4.1A). The efficacy of the pattern transfer from the PDMS stamp to the PEG hydrogel appeared to depend on the hydrogel stiffness, measured here as shear modulus (Supplementary Figure 4.1B). To ensure uniform groove patterns across all PEG hydrogels, we varied the initial groove dimensions of the PDMS stamps that were applied to hydrogels of varying stiffnesses. Doing so, we showed uniform groove depth and width on the surface of all PEG hydrogels developed here, with groove depth and width of $4 \pm 2 \mu\text{m}$ and $39 \pm 5 \mu\text{m}$ respectively (Figure 4.2B). Taking a cross section of the surface of our materials, a clear definition of grooves was visible on the PDMS stamps whereas a wrinkle-like surface

topography was visible on the PEG hydrogels (Supplementary Figure 4.1C), regardless of formulation. Still, despite the PDMS overlay during photo-crosslinking, surface patterning did not impact the polymerization of PEG hydrogels which was carried out to completion (Supplementary Figure 4.2A). In comparison, Matrigel, the standard substrate used in iPSC culture and iPSC-CM differentiation [2], was not amenable to modification of its surface topography using this patterning approach (Supplementary Figure 4.2B).

4.4.2 PEG Hydrogel Surface Topography Influenced Initial iPSC-Derived Cardiac Progenitor Cell Behavior

PEG hydrogel surface topography appeared to have a short-term influence on iPSC-CPC behavior. iPSC-CPCs seeded on patterned PEG hydrogels demonstrated adhesion and survival in elongated, aligned aggregates (Figure 4.3A). This initial alignment was rapidly lost as cells further aggregated over the course of the differentiation (Figure 4.3A). Quantifying these observations by measuring confluency levels, we observed differences in confluency during the first few days in culture across substrates with no pattern and those patterned (Figure 4.3B). Confluency levels appeared to stabilize in the later days in culture as cells aggregated across all conditions (Figure 4.3B). In comparison, when seeding fibroblasts, a cell type generally known to align easily in culture when directed to do so [25,26], these fibroblasts aligned and remained aligned over a longer period of time than our CM differentiation timeline (Supplementary Figure 4.3).

PEG hydrogel surface topography resulted in initial iPSC-CPC alignment at the cellular and aggregate level. Seeding iPSC-CPCs on a non-patterned hydrogel surface

resulted in rapid aggregation of cells across the hydrogel surface (Figure 4.4A). Instead, seeding iPSC-CPCs on a patterned hydrogel surface resulted in initial alignment of these cells at the single cell level according to the direction of the pattern axis (Figure 4.4B, Supplementary Figure 4.4). This cell alignment was visible rapidly after cell seeding but was short-lived as cells appeared to lose this alignment as they also aggregated by hour 24 (Supplementary Figure 4.4–4.5). Within 24 hours in culture, cells appeared to have aggregated according to the defined pattern axis (Figure 4.5A). Taking a cross-section of our stacked images, the wrinkled surface pattern was visible from the presence of live and dead cells (Hoechst 33342 stain) (Figure 4.5B). From this, the live cell aggregates (CellTracker Red stain) appeared to reside within the bottom curvature of our wrinkle-like pattern (Figure 4.5C). Further, the length of the cell aggregates on patterned hydrogels, as measured perpendicular to the axis of the pattern, was consistent with the hydrogel groove width (Figure 4.5C). In comparison, the length of the cell aggregates on patterned and non-patterned hydrogels was similar when measured parallel to the patterned axis (Figure 4.5C). Cell aggregation was visible over the course of 24-hour timelapse imaging in later days in culture (day 2 to 3), as cells appeared to aggregate within their respective groove, along the defined pattern axis (Supplementary Movie 4.1).

Cell seeding density was manipulated in an effort to expand the influence of our PEG hydrogel surface topography on iPSC-CPCs over time. A longer pattern retention on the cell aggregates was visible when increasing the iPSC-CPC seeding density by 50 % (Figure 4.6A). Despite continued aggregation over time in culture, aggregate alignment appeared to last until day 5 rather than day 1 in culture for cells plated at a

higher seeding density (Figure 4.6). Confluency was unchanged across the non-patterned hydrogel with original cell seeding density and the patterned hydrogels with increased cell seeding density (Figure 4.6B). With this higher seeding density, similar culture conditions as those of non-patterned hydrogels have essentially been re-created here in the context of patterned hydrogels. The remainder of this study was carried out using a cell seeding density of 90,000 cells/cm². Cell aggregate alignment was visible on the patterned PEG hydrogels over the course of the differentiation, compared to the non-patterned conditions (Figure 4.7A-B). The full-width-at-half-max (FWHM) and center-of-peak measurements were consistent across almost all timepoints during the differentiation (days 1 to 8 in culture), demonstrating durable alignment throughout most of the differentiation process (Figure 4.7C).

4.4.3 PEG Hydrogel Surface Topography Had a Limited Effect on iPSC-Derived Cardiomyocyte Protein Expression But a Clear Influence on Intracellular Organization

Protein expression remained mostly unchanged with respect to surface topography of the cell culture substrate. Seeing visible alignment of iPSC-CPCs on patterned PEG hydrogels during CM differentiation (Figure 4.6-Figure 4.7), cells were characterized to study any changes during the differentiation and maturation process (11 days and 25 days in culture, respectively). The expression of the CM differentiation marker cTnT was unchanged across all conditions tested, regardless of hydrogel stiffness and surface pattern (Figure 4.8A). The expression of the CM maturation marker MLC2v was increased for iPSC-CMs cultured on one hit PEG hydrogel (7 mM cRGDfC 3 mM

CIKVAV 2 kPa) and one higher stiffness control PEG hydrogel (3.5 mM cRGDfC 6 kPa) with 50 μ m grooves as surface patterns, when compared to their non-patterned equivalent (Figure 4.8B). However, despite an increase in MLC2v-expressing cells on these patterned conditions, the MLC2v-expressing cell population was still similar to the non-patterned condition for our other hit PEG hydrogel (3.5 mM cRGDfC 0.5 kPa) (Figure 4.8B). Similarly, fluorescent imaging further indicated largely unchanged protein expression across the various substrate conditions (Figure 4.9, Supplementary Figure 4.6-4.7). Unchanged expression of RYR2 and Connexin-43 (Cx43), additional CM maturation markers, was also visible across all conditions tested here (Supplementary Figure 4.8). Cell number, although apparently dependent on hydrogel stiffness, was unchanged with respect to varying surface patterns (Supplementary Figure 4.8). Despite the loss of clear cell aggregate alignment over the course of the differentiation, focal adhesion and iPSC-CM differentiation marker expression were unchanged throughout various differentiation timepoints (Supplementary Figure 4.9).

In a stark contrast to trends in protein expression, the orientation of intracellular proteins and nuclei appeared highly dependent on surface pattern. Fluorescent imaging of cTnT revealed highly elongated and organized myofibrils for iPSC-CMs cultured on patterned hydrogels (Figure 4.10A). Instead, cTnT appeared limited to a rounded morphology seen in iPSC-CMs cultured on non-patterned hydrogels (Figure 4.10A). Similarly, nuclear elongation and alignment was visible for cells cultured on surface patterned PEG hydrogels, whereas rounded nuclei were observed on non-patterned PEG hydrogel surfaces (Figure 4.10B).

4.5 Discussion

The synthetic PEG hydrogels fabricated here were easily modifiable to demonstrate a surface topography of interest. In comparison, Matrigel was not amenable to surface patterning using this technique (Supplementary Figure 4.2B). Other studies have used Matrigel to study the effect of guided cell organization on iPSC-CM maturation but were limited to using microcontact printing to define a desired pattern [27-29]. This technique, although successful in its ability to direct cell alignment along the axis of the edge of the pattern, was not amenable to studying the effect of micro-scale topographical cues on iPSC-CM behavior. Here, we used a photolithography approach followed by PDMS stamping of our PEG hydrogels to transfer a defined micro-pattern to our PEG hydrogels (Figure 4.1). This was unsuccessful on Matrigel, possibly due to the extremely low stiffness of this material [30], rendering pattern retention practically impossible. Other synthetic materials have been modified to demonstrate a defined microscale topography [31-34]. These studies have often been limited to using silicone-based materials (e.g. PDMS, silicon substrate) to focus on the effect of surface topography on cell behavior, however, this limits the understanding of topographical cues along with other material variables [31-34]. PEG hydrogels here supported the study of surface topography in combination with other variables (e.g. hydrogel stiffness, adhesion ligand concentration) and displayed a precise control over surface topography regardless of the other material variables (Figure 4.3B).

iPSC-CMs aggregated over time on the PEG hydrogels regardless of the confinement to a pattern. The PEG hydrogels used here resulted in iPSC-CM aggregation

over time when no pattern was present. On patterned PEG hydrogels, iPSC-CPCs aggregated within the patterned grooves but as they differentiated to iPSC-CMs the cell aggregates appeared to lose this pattern definition. This may be caused by continued aggregation over time, as cells detached from the material and relied increasingly on cell-to-cell contact, despite the presence of a physical barrier forcing alignment and elongation. Many studies looking at iPSC-CM differentiation or maturation according to material surface topography show monolayer tissue formations and therefore a continuous effect of surface topography on cell behavior over time in culture [27-29,31-36], though culture conditions are extensively different (e.g. cell seeding density, cell culture timeline, cell culture substrate, micropattern dimensions). Alvarez-Paino et al. looked at the effects of both surface topography and adhesion ligands on cell aggregate formation and showed that the surface chemistry had a more important influence on cell population adhesion and survival over the surface topography [37], indicating that the presence of a physical barrier does not have a strong impact on iPSC-CM population morphology. Further, here, iPSC-CPCs adhered and elongated along the defined surface pattern but appeared to lose that elongation as they committed to iPSC-CMs (Figure 4.6A). Other studies also highlighted the importance of cell-to-cell contact for iPSC-CM culture specifically [38-40].

PEG hydrogel surface topography resulted in limited effect on iPSC-CM differentiation and maturation. This may be caused by the short-term effect of the PEG hydrogel surface topography which was largely limited to iPSC-CPC differentiation rather than iPSC-CM culture (Figure 4.6A). However, there was considerable structural

organization both at the myofibril and nuclear level (Figure 4.10), indicating that short-term iPSC-CPC alignment may have a long-term effect on iPSC-CM maturation even after 25 days in culture. To our knowledge, this was the first study showing a long-term influence of iPSC-CPC behavior on iPSC-CM behavior, through iPSC-CPC cell and tissue alignment specifically. Our results also corroborated other studies which showed limited influence of surface topography on gene and protein expression [9,27,31,32]. This suggested that post-transcriptional events may be linked to iPSC-CM intracellular organization changes as shown here.

4.6 Conclusion

Successful manipulation of PEG hydrogels such that they demonstrate necessary cues for a desired cell behavior is a crucial aspect of efficient cell manufacturing for downstream applications. iPSC-CM maturation is highly dependent on the cell's microenvironment, including topographical cues. Here, the PEG hydrogel surface was successfully manipulated to present a defined pattern. The PEG hydrogel surface topography successfully directed cell and aggregate alignment, over a short period of time. This short-term cell and aggregate alignment had minimal influence on protein expression but demonstrated a direct influence on intracellular and intercellular organization. These results highlight the importance of substrate manipulation at all stages of cell culture and the long-term influence of substrates on cell behavior.

4.7 Acknowledgements

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4.8 References

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4.9 Figures

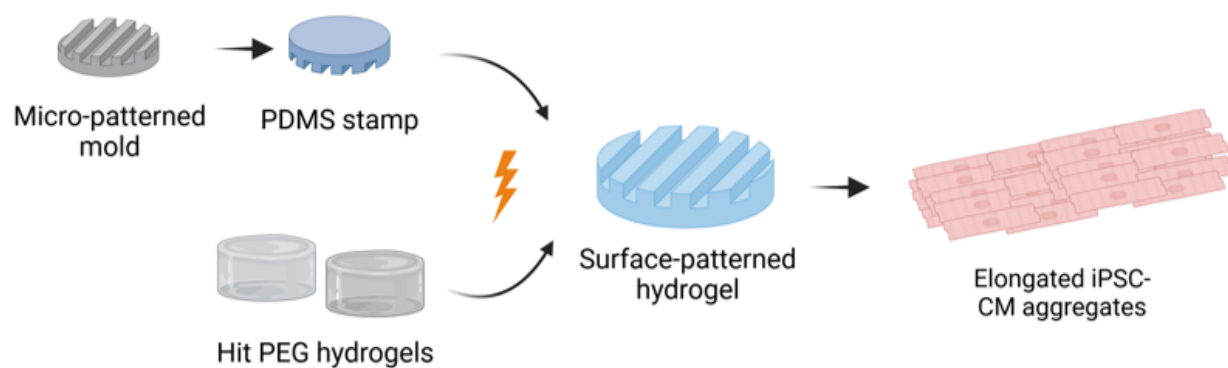


Figure 4.1. Schematic overview of PEG hydrogel patterning.

Schematic representation of PEG hydrogel surface patterning workflow. Schematic not to scale.

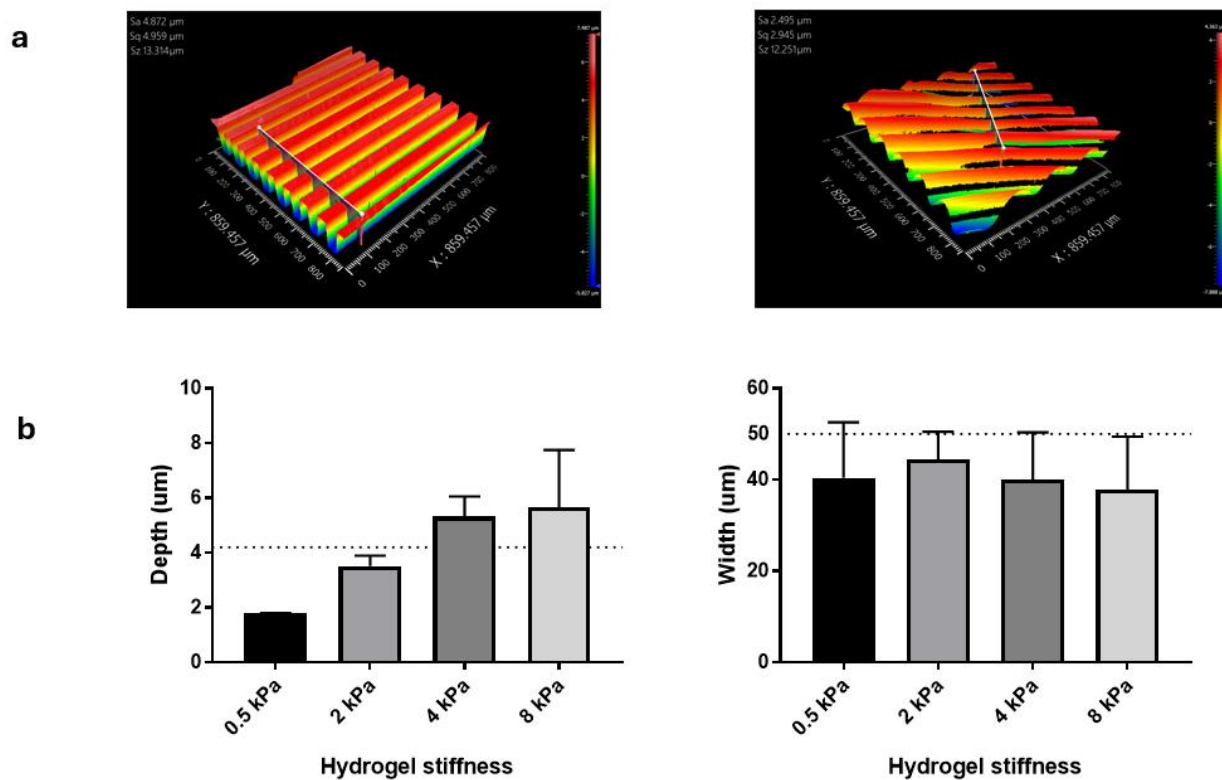


Figure 4.2. Surface patterning of PDMS and PEG hydrogel resulted in clearly defined pattern on substrate surface.

(A) Optical profilometry images of PDMS substrate (left) and PEG hydrogel (right) demonstrating defined surface pattern. **(B)** Depth and width measurements of grooves patterned on PEG hydrogel surface using PDMS molds of varying pattern depth.

Figure 4.3 PEG hydrogel surface patterning resulted in decreased cell confluency in initial days post-seeding compared to smooth surface hydrogels. (A) Representative images of live cells on patterned PEG hydrogel. (B) Quantification of confluency on PEG hydrogels of varying surface topography.

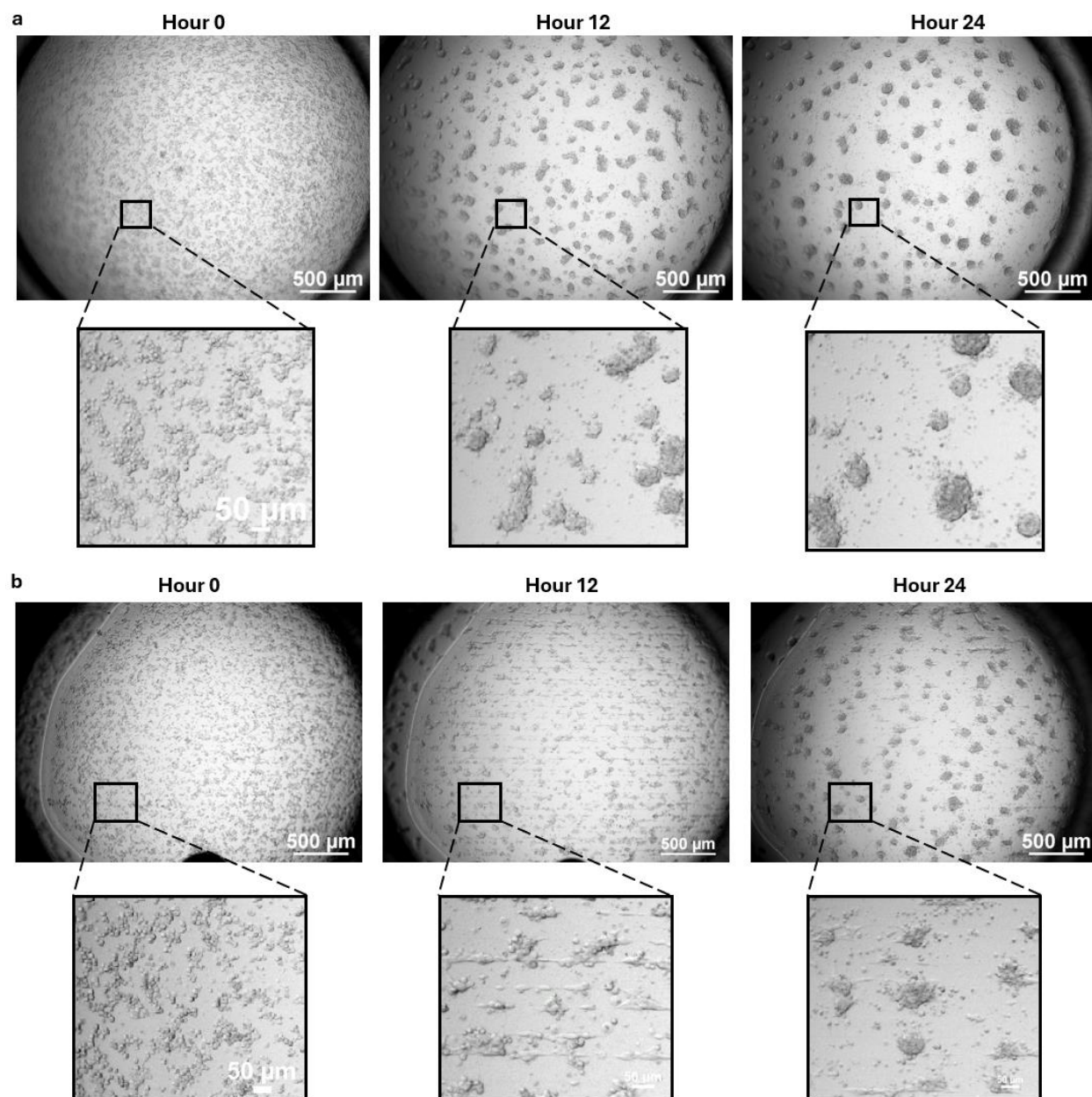


Figure 4.4 iPSC-CPCs aligned at the cellular level along directed pattern axis over several hours after seeding.

Representative brightfield images of iPSC-CPCs over 24 hours on the surface of **(A)** unpatterned PEG hydrogel, and **(B)** patterned PEG hydrogel.

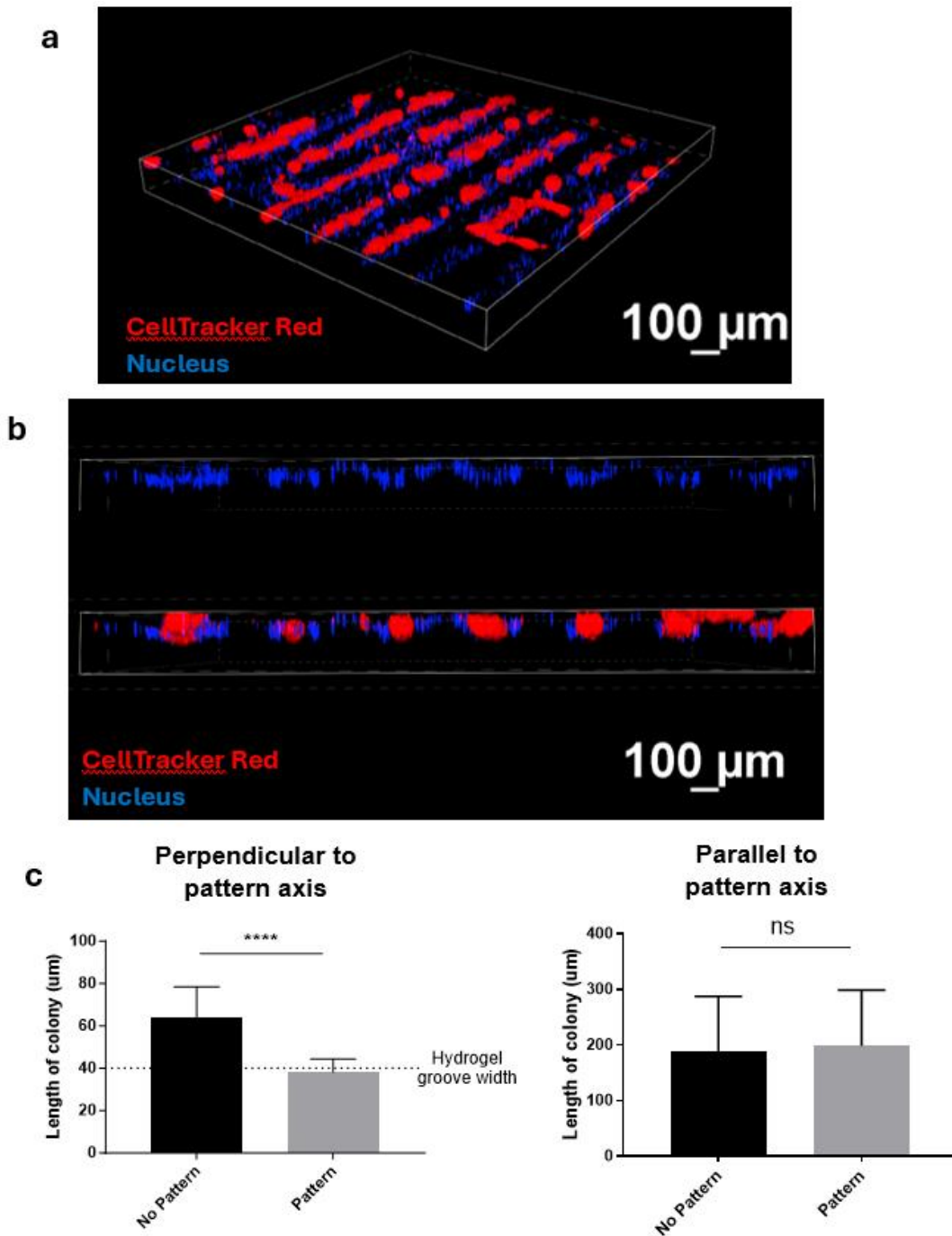


Figure 4.5. iPSC-CPC aggregates resided within patterned grooves on PEG hydrogel surface after 24 hours in culture.

(A) Fluorescent images of iPSC-CPCs aggregate volumes. Blue: Hoechst 33342, Red: CellTracker Red. **(B)** Fluorescent images of cross sections of iPSC-CM aggregates. **(C)** Measurements of iPSC-CPC aggregate width on patterned and non-patterned PEG hydrogels.

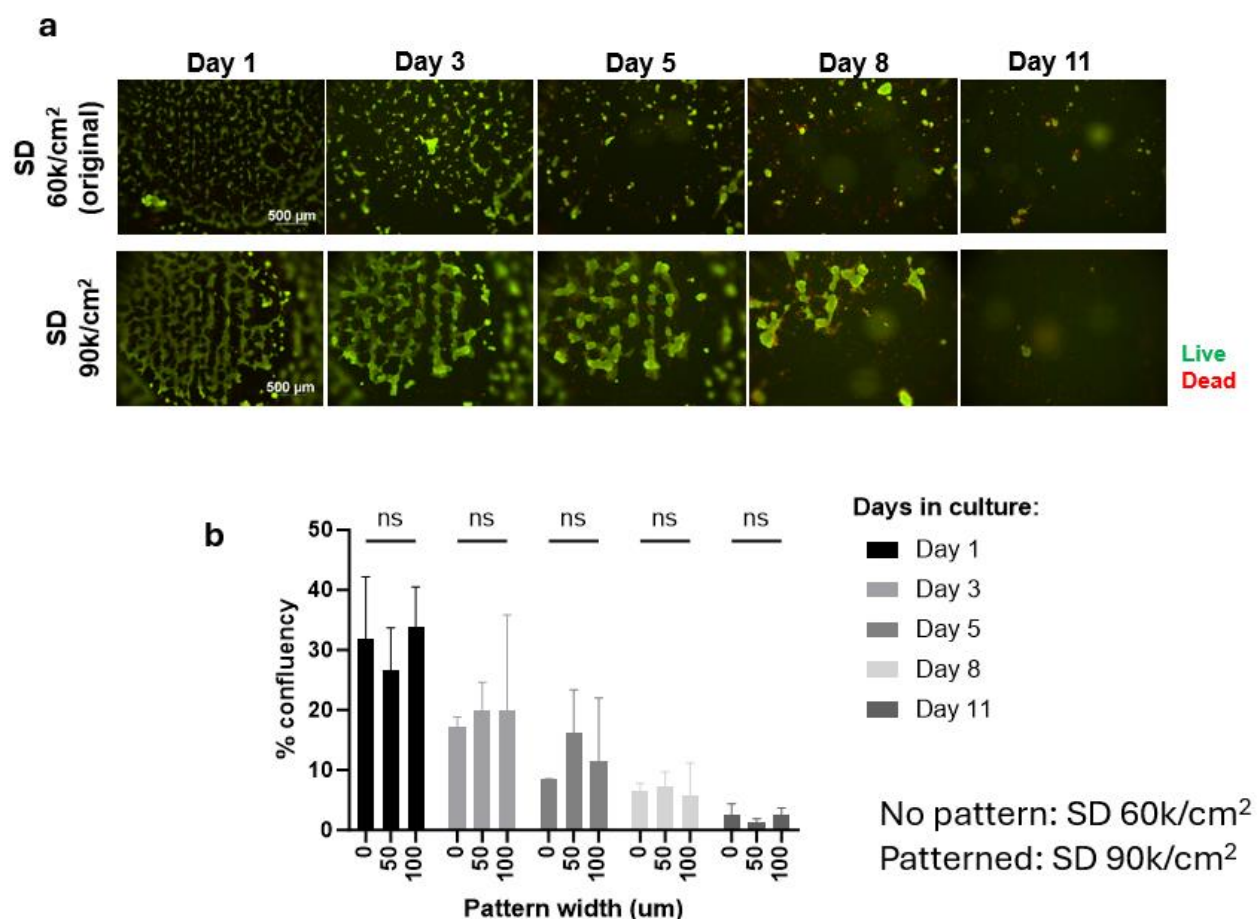


Figure 4.6. Increased iPSC-CPC seeding density yielded longer lasting cell aggregate alignment and higher cell confluency. (A) Representative images of live cells on patterned PEG hydrogel. **(B)** Quantification of confluency on PEG hydrogels of varying surface topography.

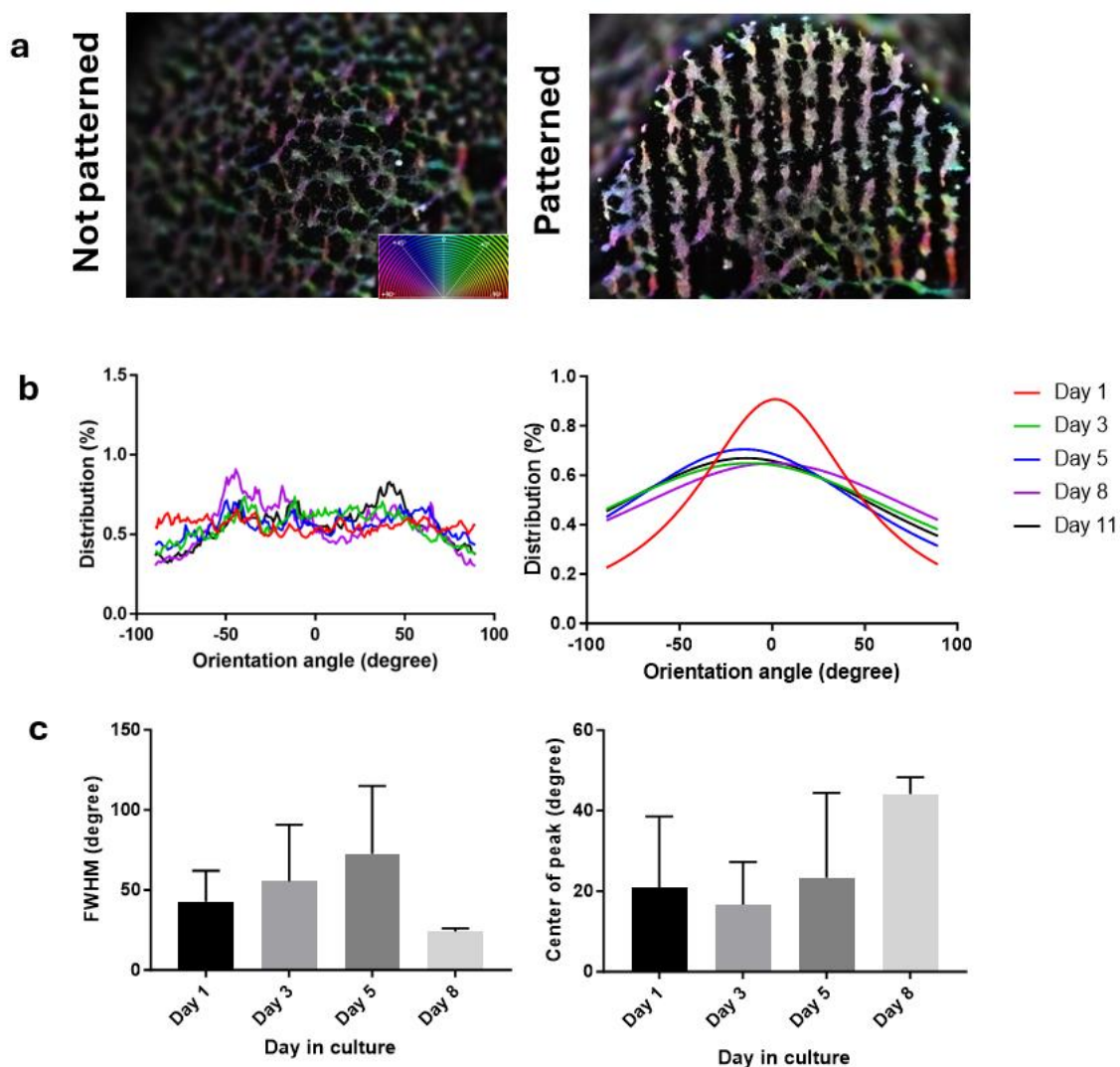


Figure 4.7. Patterned PEG hydrogels resulted in short term alignment of cell aggregates during iPSC-CPC to -CM differentiation.

(A) Representative color maps of iPSC-CM aggregate orientation with respect to PEG hydrogel surface topography. **(B)** Distribution of orientation angle of iPSC-CM aggregates over time. **(C)** Full width at half maximum (FWHM) and center of peak of orientation angle distribution of iPSC-CM aggregates.

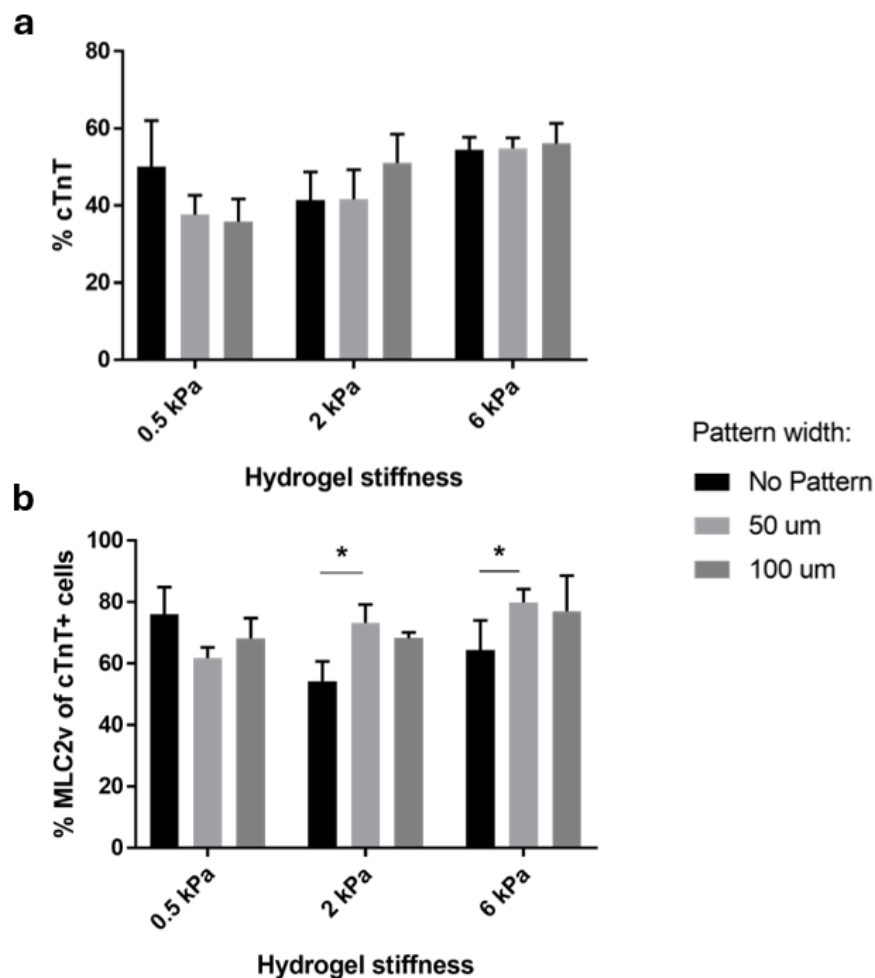


Figure 4.8. PEG hydrogel surface patterning influenced MLC2v expression on specific PEG hydrogel conditions but left cTnT expression unchanged.

(A) Flow cytometry quantification of cTnT on various substrate conditions. **(B)** Flow cytometry quantification of MLC2v for cTnT-expressing cells on various substrate conditions.

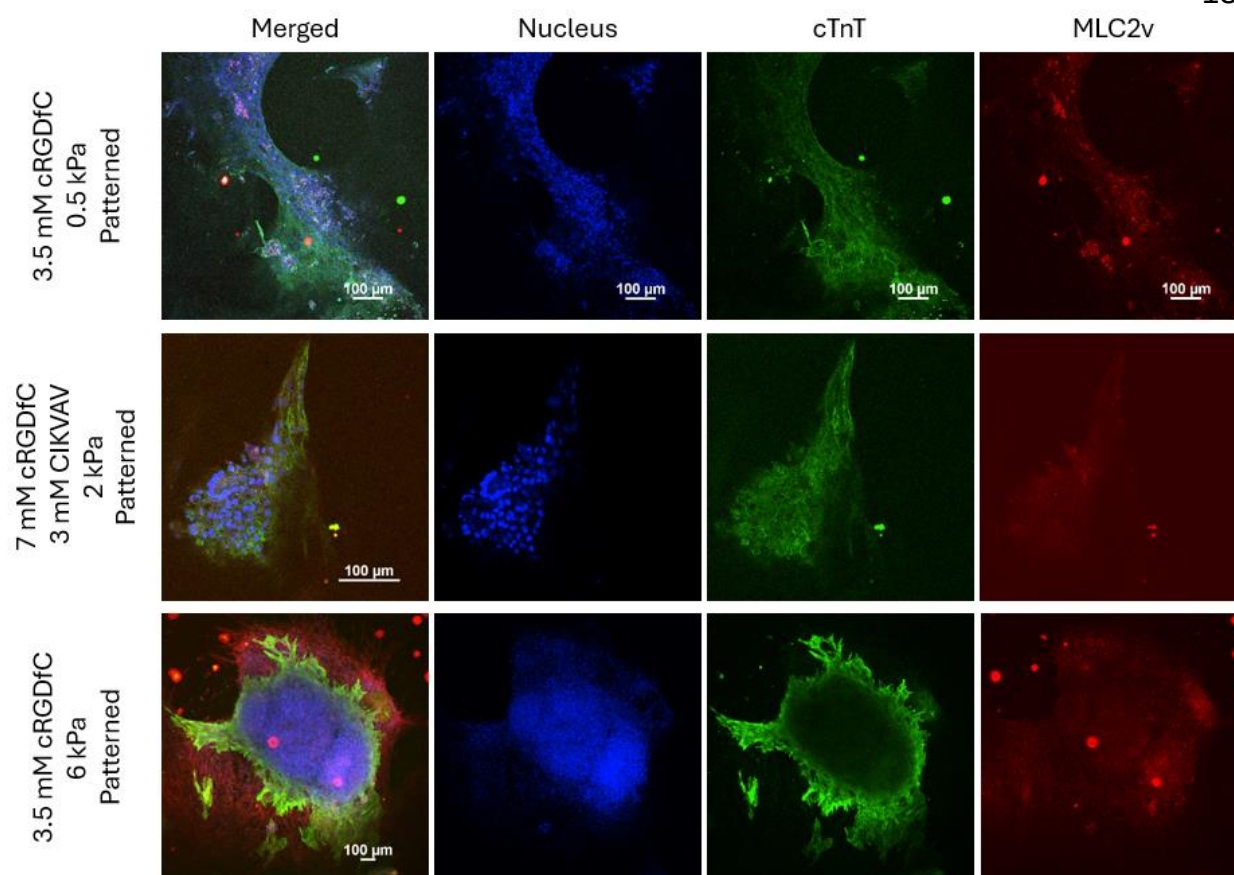


Figure 4.9. PEG hydrogel surface topography showed limited influence on expression of maturation markers.

Representative fluorescence images of iPSC-CM maturation markers on varying substrates.

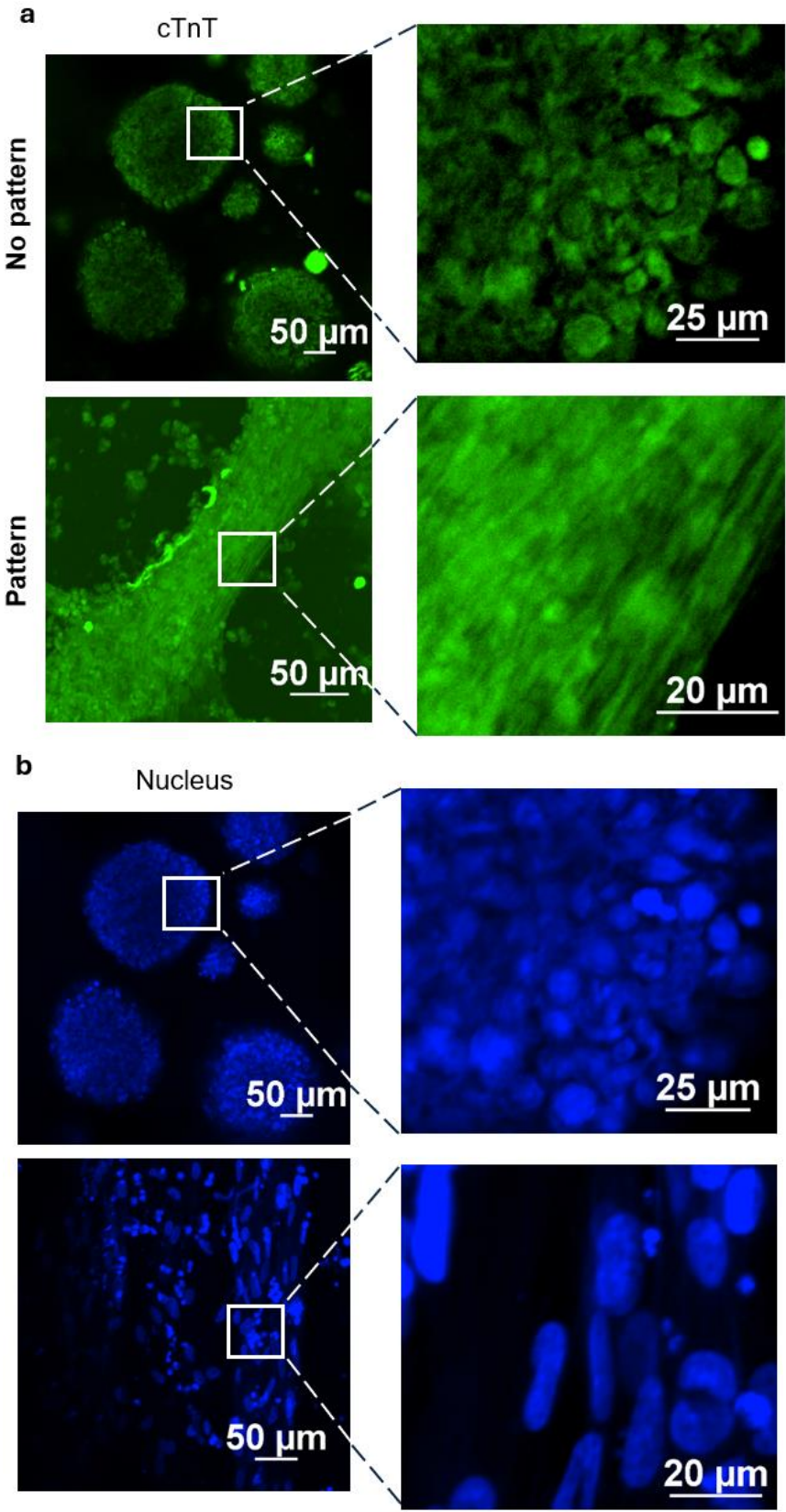
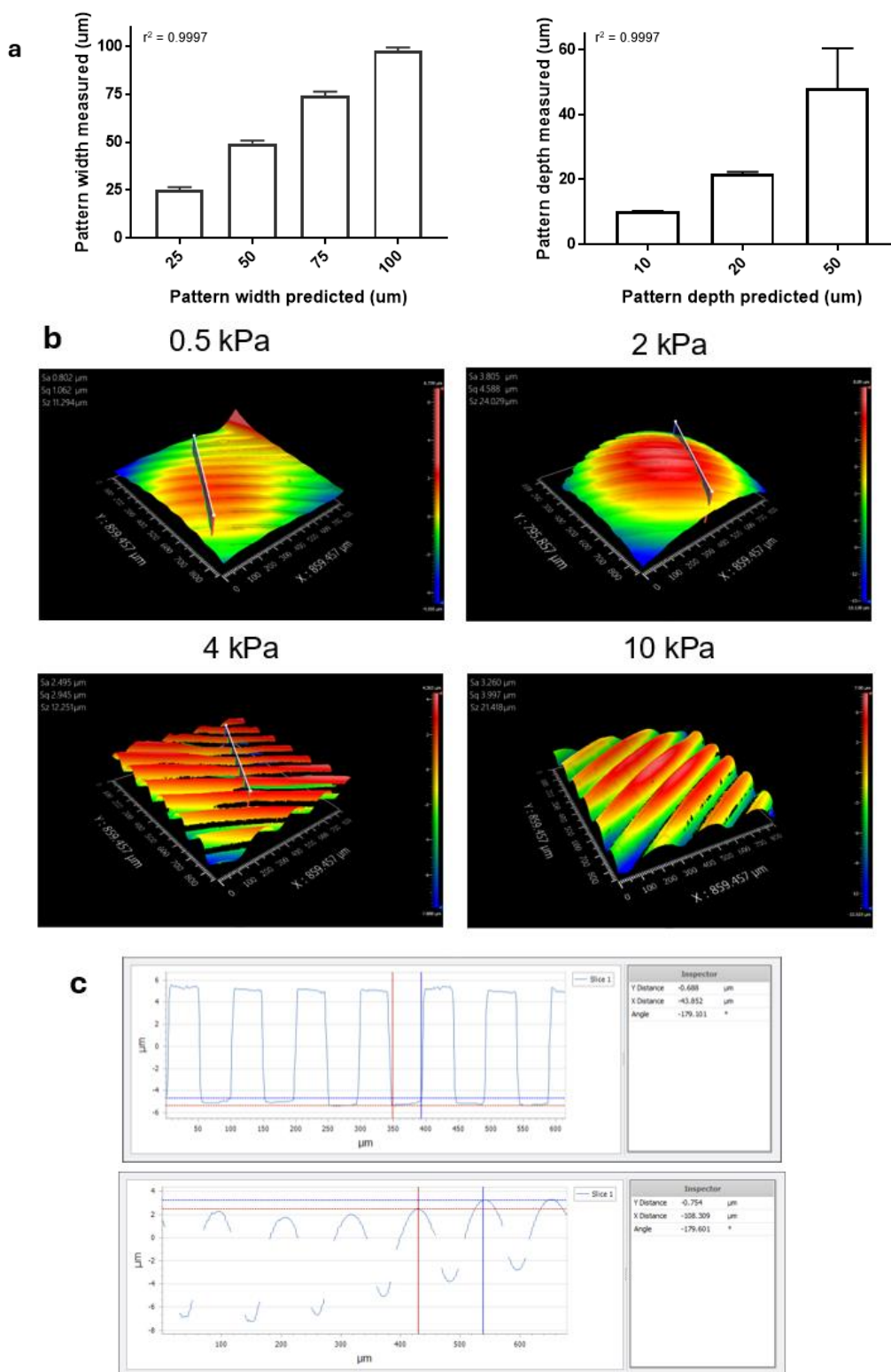


Figure 4.10. Surface patterning of PEG hydrogels resulted in myofibril and nuclear alignment.

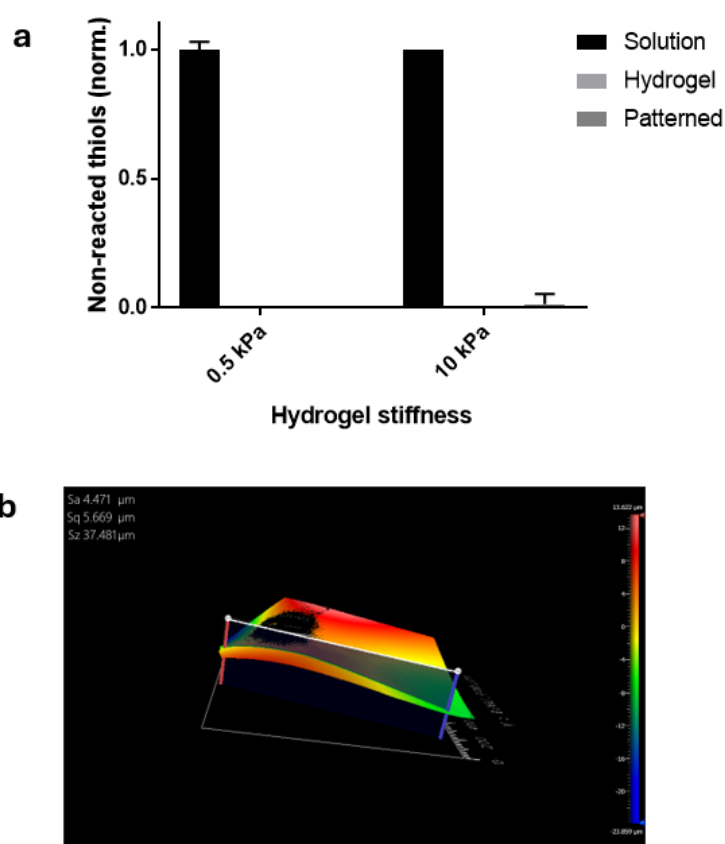
(A) Representative fluorescence images of cTnT expression for iPSC-CMs cultured on substrates with varying surface topography. **(B)** Representative fluorescence images of iPSC-CM nucleus on substrates with varying surface topography.

4.10 Supplementary Figures



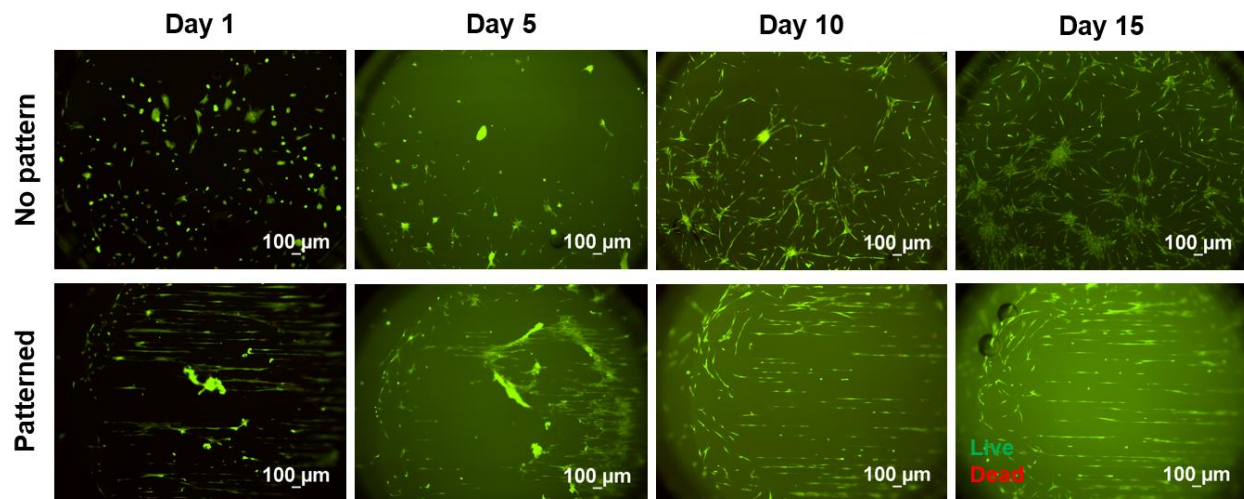
Supplementary Figure 4.1. Surface patterning offered precise control over substrate surface topography profile.

(A) Optical profilometry measurements of PDMS stamps presenting various surface groove widths and depths. **(B)** Optical profilometry images of PEG hydrogels with varying stiffnesses. **(C)** Cross section representation of surface profile of PDMS (top) and PEG hydrogel (bottom).

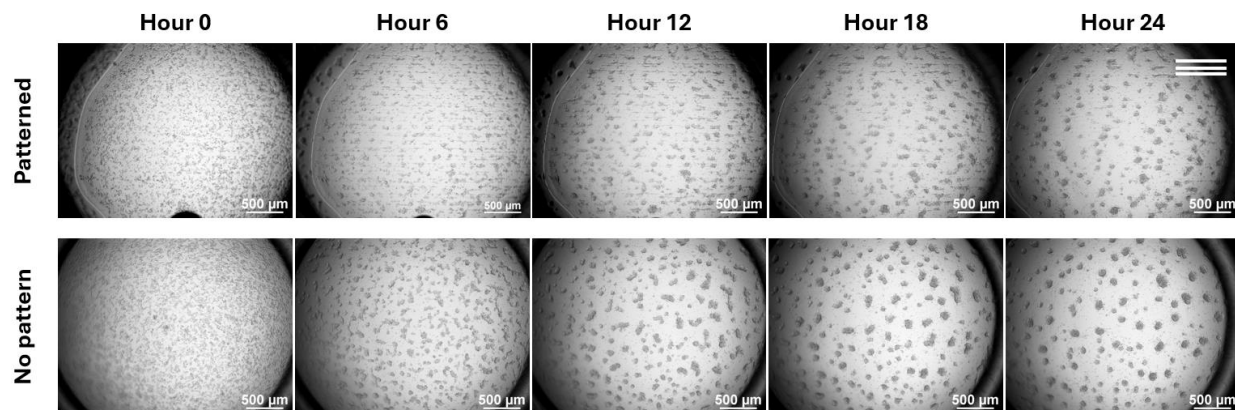


Supplementary Figure 4.2. Material characterization after surface patterning.

Surface stamping did not prevent **(A)** polymerization of PEG hydrogels, or **(B)** the gelling process of Matrigel although it also does not yield a patterned surface on Matrigel.

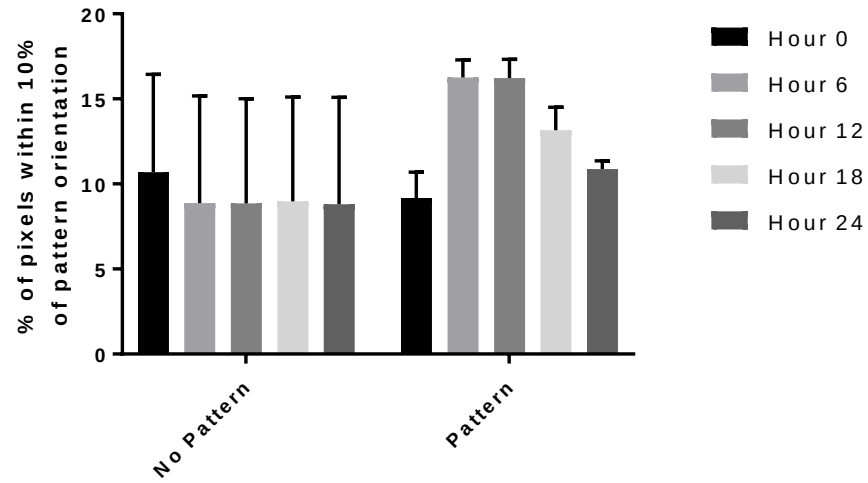


Supplementary Figure 4.3. Fibroblasts aligned and remained aligned over time on patterned PEG hydrogel surface.

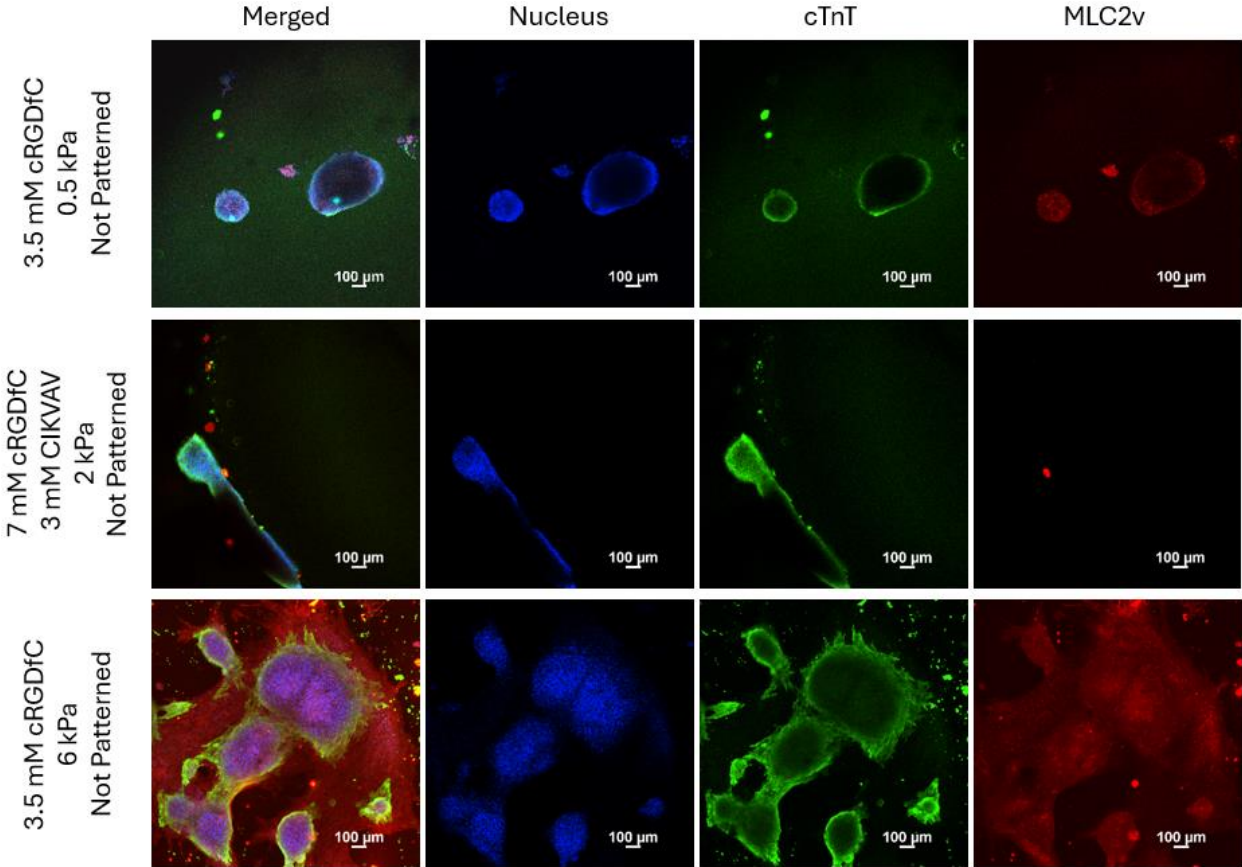


Supplementary Figure 4.4. iPSC-CPCs aligned at the cellular level along directed pattern axis over several hours after seeding.

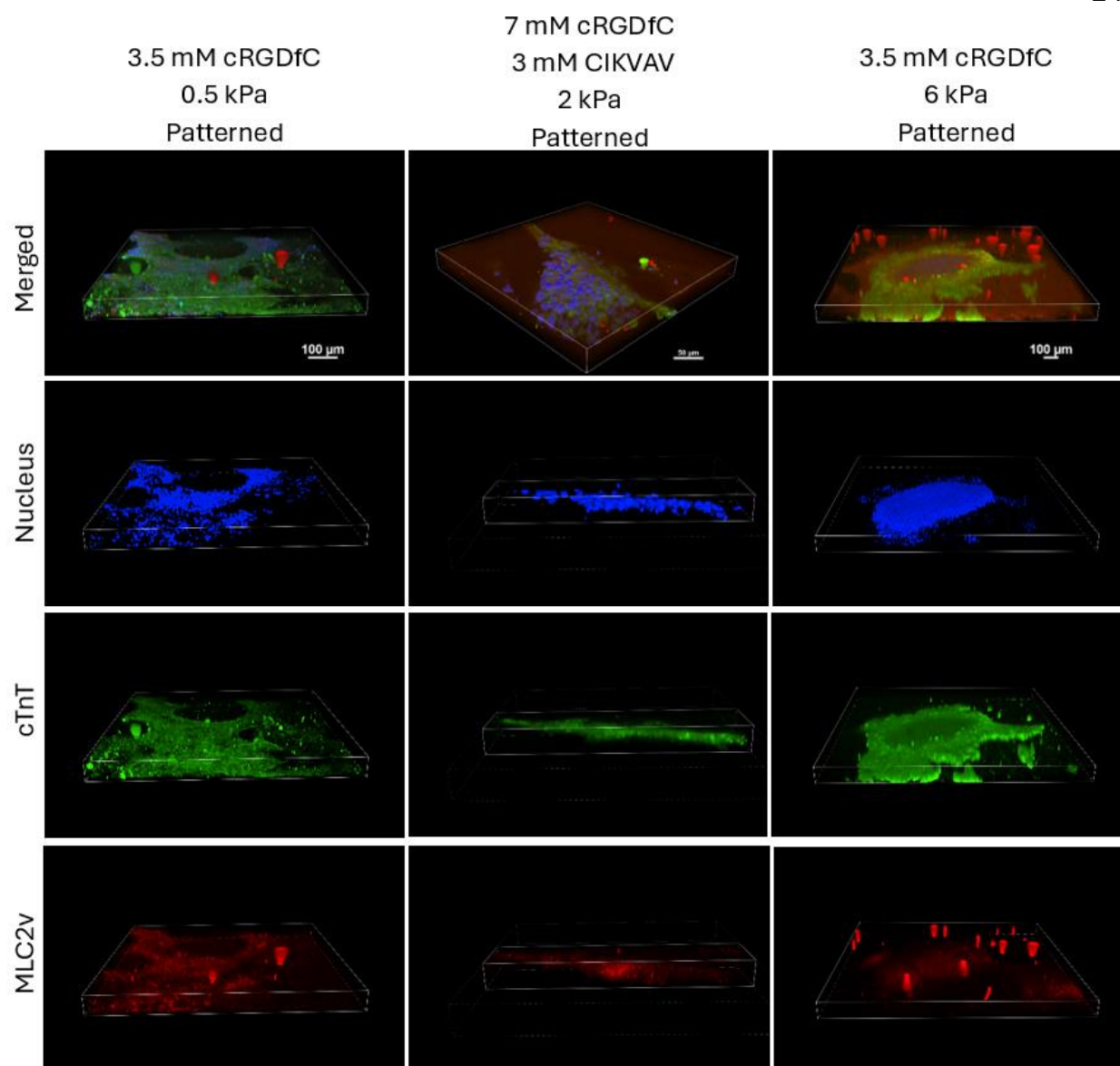
Representative brightfield images of iPSC-CPCs over 24 hours on varying surface topographies.



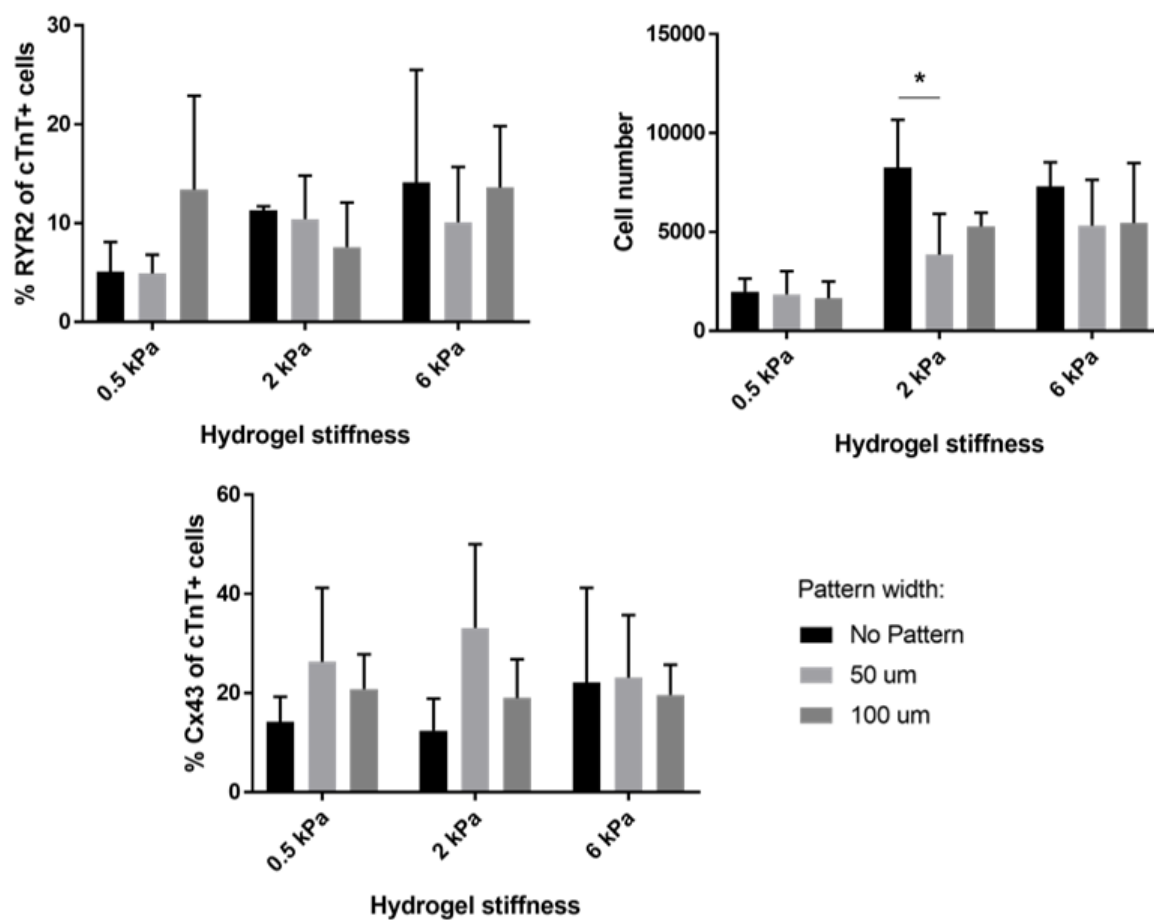
Supplementary Figure 4.5. Patterned PEG hydrogels resulted in short term alignment of cell aggregates during iPSC-CPC to –CM differentiation.



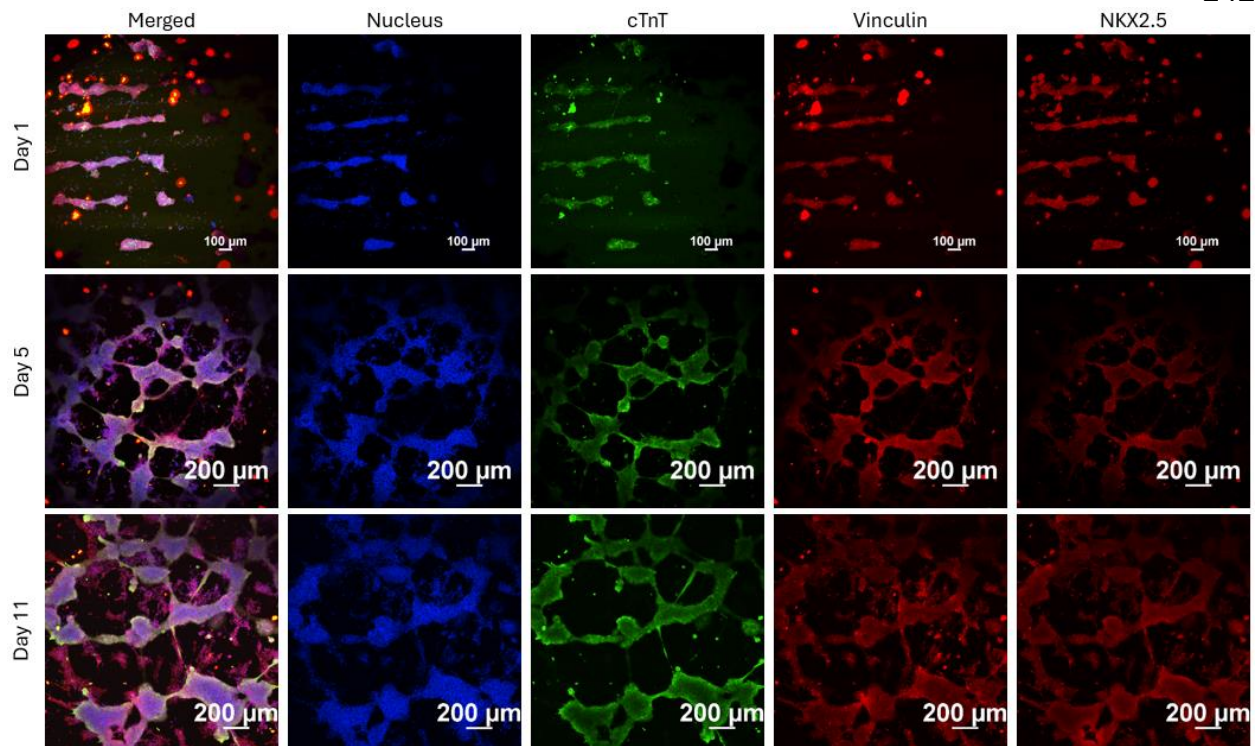
Supplementary Figure 4.7. Representative fluorescence images of iPSC-CM maturation markers on varying substrates.



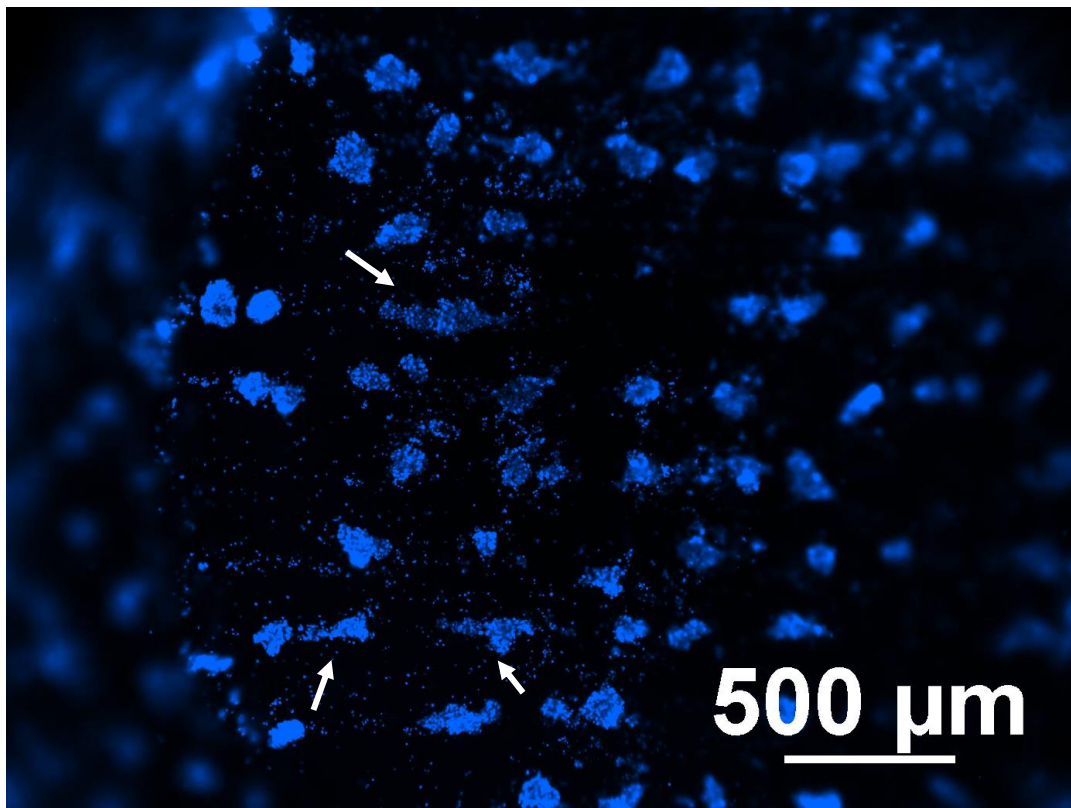
Supplementary Figure 4.8. Representative fluorescence z-stack images of iPSC-CM maturation markers on varying substrates.



Supplementary Figure 4.6. Flow cytometry quantification of Ryanodine receptor-2, Connexin-43, and cell number for cells cultured on varying substrates.



Supplementary Figure 4.9. Representative fluorescence images of iPSC-CM differentiation markers over the course of the differentiation timeline on surface patterned PEG hydrogels.



Supplementary Movie 4.1. iPSC-CPCs seeded on patterned PEG hydrogel surface aggregated within defined grooves during days 2 to 3 after seeding.

Chapter 5 : Optical Redox Imaging to Screen Synthetic Hydrogels for iPSC-Derived Cardiomyocyte Differentiation and Maturation

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* Authors contributed equally to this work: MJA – iPSC culture and maintenance, iPSC-derived cardiomyocyte differentiation, PEG hydrogel synthesis, experimental design, data analysis, manuscript writing; DED – iPSC-derived cardiomyocyte differentiation, fluorescence lifetime imaging, widefield optical redox imaging, experimental design, data analysis, manuscript writing.

5.1 Abstract

Significance: Heart disease is the leading cause of death in the United States, yet research is limited by the inability to culture primary cardiac cells. Cardiomyocytes (CMs) derived from human induced pluripotent stem cells (iPSCs) are a promising solution for drug screening and disease modeling.

Aim: Induced pluripotent stem cell-derived CM (iPSC-CM) differentiation and maturation studies typically use heterogeneous substrates for growth and destructive verification methods. Reproducible, tunable substrates and touch-free monitoring are needed to identify ideal conditions to produce homogenous, functional CMs.

Approach: We generated synthetic polyethylene glycol-based hydrogels for iPSC CM differentiation and maturation. Peptide concentrations, combinations, and gel stiffness were tuned independently. Label-free optical redox imaging (ORI) was performed on a widefield microscope in a 96-well screen of gel formulations. We performed live-cell

imaging throughout differentiation and early to late maturation to identify key metabolic shifts.

Results: Label-free ORI confirmed the expected metabolic shifts toward oxidative phosphorylation throughout the differentiation and maturation processes of iPSC CMs on synthetic hydrogels. Furthermore, ORI distinguished high and low differentiation efficiency cell batches in the cardiac progenitor stage.

Conclusions: We established a workflow for medium throughput screening of synthetic hydrogel conditions with the ability to perform repeated live-cell measurements and confirm expected metabolic shifts. These methods have implications for reproducible iPSC-CM generation in biomanufacturing.

5.2 Introduction

Cardiovascular disease remains the leading cause of death in the United States [1] and costs the healthcare system over \$200 billion annually [2]. Heart diseases remain difficult to treat and study both due to the lack of regenerative potential in the human heart and the inability to maintain primary cardiomyocytes (CMs; contractile heart muscle cells) in culture. Human induced pluripotent stem cell-derived CMs (iPSC-CMs) are of great interest to the biomanufacturing industry, with promising applications that include improved drug screening platforms, accurate disease models, and personalized regenerative medicine. iPSCs can be effectively reprogrammed to generate high-purity CM batches [3,4]. However, in vitro maturation of iPSC-CMs remains difficult, with cells demonstrating a fetal-like phenotype characterized by disorganized structure and decreased electrophysiological capabilities [5,6]. Stem cell-derived CMs also exhibit

batch-to-batch and line-to-line variability in function and structure [7–11]. Furthermore, methods to assess differentiation and maturation efficiency (e.g., ribonucleic acid (RNA) sequencing, immunostaining) are generally destructive and low-throughput [12,13]. The efficient production and widespread use of iPSC-CMs will require consistent manufacturing of functionally mature cell batches and touch free, higher throughput, and nondestructive monitoring.

Various techniques have been employed to improve all facets (e.g., bioenergetic, structural, electromechanical) of iPSC-CM maturation; these include electromechanical stimulation, extended culture, media additives, and co-culture with other cell types [14,15]. Culture substrate is one critical regulator of cell behavior in vitro. For example, the composition (e.g., extracellular matrix proteins, synthetic polymers) and stiffness of the substrate affect the motility [16–18] and morphology [18–20] of many cell types. Substrate properties also impact stem cell differentiation and maturation potential [20–24], and synthetic hydrogels with tunable physical, mechanical, and chemical properties [25,26] present a method for regulating stem cell fate in culture. Synthetic substrates show promise for iPSC culture and targeted differentiations [14,25,27,28] although improvements are needed in terms of maturation. iPSC-CM culture has traditionally relied on Matrigel, a mixture of basement membrane proteins derived from murine sarcomas. However, the composition, mechanical properties, and potential for xenogenic contamination vary among Matrigel batches, and it cannot be manipulated to fine-tune substrate properties and control degradation [25,27,29]. Synthetic substrates, therefore, may be an attractive alternative for robust iPSC-CM manufacturing.

Verifying successful iPSC-CM differentiation and/or maturation on any substrate typically relies on techniques such as quantitative polymerase chain reaction (qPCR), immunostaining, and flow cytometry for cardiac markers [3,12,13]. While highly specific, these methods are destructive and can therefore only be performed at a single timepoint. Label-free optical imaging and sensing techniques are attractive touch-free solutions for repeated monitoring during cellular development [30]. Different modalities can detect the metabolic, organizational, and structural changes that occur during stem cell differentiation and subsequent maturation of the resulting cell types. Optical redox imaging (ORI) makes use of the intrinsic fluorescent properties of reduced nicotinamide adenine dinucleotide (phosphate) [NAD(P)H] and oxidized flavin adenine dinucleotide (FAD), two coenzymes involved in hundreds of cellular metabolic reactions [31–34]. Multiphoton imaging of NAD(P)H and FAD fluorescence intensities has been used to quantify changes that occur during mesenchymal stem cell differentiation into chondrocytes [35], osteocytes [35,36], and adipocytes [37–39]. In addition, the fluorescence lifetimes of NAD(P)H and FAD are distinct in their protein-bound and free states and provide information on environmental factors (e.g., pH, oxygen) [33,34,40,41]. Label-free fluorescence lifetime imaging microscopy (FLIM) has been used to distinguish undifferentiated stem cells and progenitors from their differentiated counterparts [42–45], separate high and low differentiation efficiency groups [46], and perform long-term monitoring during maturation [47,48]. Other label-free imaging modalities can monitor structural changes that occur during stem cell differentiation [49–51]. Importantly, these

techniques are repeatable and live cell-compatible, allowing traditional endpoint analyses to be performed on the same cell batches.

iPSCs primarily use glycolysis for adenosine triphosphate (ATP) production and shift to oxidative phosphorylation during differentiation to CMs [52,53], following the metabolic trends of fetal and adult CMs, respectively [54]. Here, we used label-free optical imaging of NAD(P)H and FAD to verify these expected metabolic changes in cells developing on synthetic hydrogels. Polyethylene glycol (PEG)-based hydrogels were crosslinked with fibronectin and laminin derived peptides and tuned to relevant physiological stiffnesses. We used autofluorescence FLIM to verify increased oxidative metabolism and NAD(P)H protein binding activity throughout differentiation. To increase throughput and screen more hydrogel formulations, we next used widefield NAD(P)H and FAD autofluorescence imaging to calculate the optical redox ratio (ORR, defined as the fluorescence intensity ratio of $\text{NAD(P)H} / (\text{NAD(P)H} + \text{FAD})$ at multiple timepoints throughout differentiation. We found that ORR decreased in cells cultured on all synthetic gel formulations during differentiation, early maturation (days 16 to 30) and late maturation (day 30+), indicating a decline in glycolytic metabolism and an increase in oxidative phosphorylation. Finally, we observed metabolic differences in high and low differentiation efficiency cells, with groups exhibiting significantly different ORR in the cardiac progenitor stage. These results suggest that widefield autofluorescence ORI can be used repeatedly during iPSC-CM differentiation and maturation on synthetic hydrogels to monitor cell metabolism and may be a useful screening tool for cell manufacturing.

5.3 Methods

5.3.1 Synthetic Hydrogels

Hydrogels were prepared using 8-arm 20 kDa norbornene-functionalized PEG (JenKem Technology, Plano, Texas, United States), 3.4 kDa PEG di-thiol (Laysan Bio, Arab, Alabama, United States) crosslinker, 0.1 wt/wt% Irgacure 2959 (CIBA, Basel, Switzerland) photoinitiator, diluted with phosphate-buffered saline (PBS) to reach desired concentrations. The following peptides were added at varying concentrations: CRGDSP (referred to as L), head-to-tail cyclized RGDfC (cRGDfC, or C), CIKVAV (I), and CYIGSR (Y) (Supplementary Table 5.1). All peptides were purchased from GenScript. Here, we developed hydrogels demonstrating physiologically relevant stiffnesses, corresponding to neonatal (0.5 and 2 kPa), healthy adult (4 and 6 kPa), and fibrotic adult (10 kPa) cardiac tissue [55]. Hydrogel formulations are written as peptide concentration in mM, single-letter peptide abbreviation, and stiffness in kPa (e.g., 7 mM cRGDfC 3 mM CIKVAV 2 kPa is written 7C-3I 2). Hydrogel precursor solutions were exposed to ultraviolet light (UV, 365 nm, 4.5 mW/cm², 5 min) for polymerization. Hydrogels were then incubated in RPMI 1640 medium (Gibco) at 37°C until use. All gel formations for a particular experiment (Supplementary Tables 5.1–5.6) were made in triplicate.

Matrigel (Corning, Corning, New York, United States) was used as a control substrate in three forms: Matrigel diluted in DMEM/F12 (ThermoFisher Scientific, Waltham, Massachusetts, United States) medium forming a thin plate coating (referred to as “coated plates” throughout the text), undiluted Matrigel forming a gel (“Matrigel”),

and undiluted growth factor reduced “Geltrex” forming a gel (ThermoFisher Scientific). The coated plate represents a widely used standard culture substrate for iPSC-CMs [3].

5.3.2 iPSC Maintenance

Human iPSCs (WTC-11) were maintained on coated plates and fed daily with mTeSR1 medium (StemCell Technologies, Vancouver, Canada). Cells were passaged every 3 to 4 days using Versene (ThermoFisher Scientific) and replated onto freshly coated plates in mTeSR1 supplemented with 5 μ M Y-27632 (Tocris, Bristol, United Kingdom).

5.3.3 Cardiomyocyte Differentiation

CM differentiation was performed as previously described [3]. Briefly, iPSCs were passaged using Accutase (ThermoFisher Scientific) and plated on fresh coated plates in mTeSR1 supplemented with 5 μ M Y-27632 at a cell density of 40,000 cells/cm². iPSCs were maintained for 2 days prior to differentiating. CM differentiation was initiated (day 0) by activating Wnt signaling with 7 μ M CHIR99021 (Selleckchem, Houston, Texas, United States) in RPMI 1640 (Gibco, Billings, Montana, United States) and B27 supplement without insulin (LifeTechnologies, Carlsbad, California, United States). Wnt signaling was inhibited with 5 μ M IWP2 (Tocris) in RPMI/B27 without insulin after 48 h (day 2). Cells were maintained in RPMI/B27 plus insulin (LifeTechnologies) beginning day 4.

On day 5 of differentiation, cardiac progenitor cells (CPCs) were lifted and singularized using Accutase, frozen in 60% RPMI/B27 without insulin, 30% fetal bovine serum, and 10% dimethyl sulfoxide (DMSO), and stored in liquid nitrogen. Cells were thawed as needed and replated in RPMI/B27 without insulin with 5 μ M Y-27632 at a 1:1

seeding ratio on Matrigel, Geltrex, or synthetic hydrogels in a 96-well angiogenesis plate (Ibidi, Fitchburg, Wisconsin, United States). The media was switched to RPMI/B27 plus insulin after 24 h, and cells were fed every 48 h until day 10 and every 72 h after.

5.3.4 Flow Cytometry

Cells were assessed for differentiation efficiency by cardiac troponin (cTnT) expression on day 16 and on day 30 for expression of the early maturation marker myosin light chain-2 (MLC2). Flow cytometry was performed as previously described [3]. Briefly, cells were dissociated with Accutase (ThermoFisher Scientific), fixed in 1% paraformaldehyde for 20 min at room temperature, and incubated in 90% methanol for 20 min at 4°C before storing at -20°C until staining. Cells were washed twice in flow buffer 1 (FB-1) consisting of PBS and 0.5% bovine serum albumin (ThermoFisher Scientific) and then incubated in flow buffer 2 (FB-2) consisting of FB-1 and 1% Triton-X 100 (ThermoFisher Scientific) and the primary antibody at 4°C overnight. Samples were washed with FB-2 and incubated with a secondary antibody in FB-2 at room temperature for 30 min in the dark. Cells were washed in FB-2 and resuspended in FB-1 for processing. Flow cytometry was performed on an Attune NxT (ThermoFisher Scientific), and data were analyzed using FCS Express. Primary antibodies used were cTnT (Abcam, Cambridge, United Kingdom, 1:800), MLC2a (Synaptic Systems, Göttingen, Germany, 1:200), and MLC2v (ProteinTech, Rosemont, Illinois, United States, 1:200). The secondary antibodies used were AlexaFluor 488 and 568 (Abcam, 1:1000).

5.3.5 Fluorescence Lifetime Imaging and Analysis

Multiphoton imaging was performed using a custom-built multiphoton microscope (Ultima, Bruker, Madison, Wisconsin, United States) consisting of an inverted microscope body (Ti-E, Nikon, Tokyo, Japan) coupled to an ultrafast tunable laser source (80 MHz, 100 fs pulses; Insight DS+, Spectra Physics, Santa Clara, California, United States). Images were acquired using time-correlated single-photon counting electronics (SPC 150, Becker & Hickl GmbH, Berlin, Germany) using Prairie View software (Bruker). NAD(P)H was excited at 750 nm (~8 mW at the sample) using a 20× water immersion, 1.0 NA objective (Plan Apo, Zeiss, Jena, Germany) with 1× optical zoom, 4.8 μs pixel dwell time, 60-s integration time, and image size of 512 x 512 pixels. NAD(P)H emissions were separated from excitation light using a 720 long pass filter and collected using a GaAsP photomultiplier tube (H7422, Hamamatsu, Shizuoka, Japan) and a 466/40 nm bandpass filter. A single field of view (FOV, ~300 μm×300 μm) was taken of each gel. Images were acquired on days 6, 8, 10, and 16 post differentiation. The instrument response function was measured using a second-harmonic generation signal from urea crystals excited at 890 nm, with full width at half-maximum of 240 ps.

Fluorescence lifetime components were computed for each image pixel using SPCImage (v8.0, Becker & Hickl GmbH). The background was thresholded, and the pixel-wise decay curves were fit to a biexponential model convolved with the instrument response function, using an iterative parameter optimization to obtain the lowest sum of the squared differences between the model and data (weighted least squares algorithm). The two-component model is $I(t) = \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2} + C$, where $I(t)$ is the fluorescence

intensity at time t after the laser excitation pulse, τ_1 and τ_2 are the fluorescence lifetimes of the short and long lifetime components, respectively, α_1 and α_2 are the fractional contributions of the short and long lifetime components, respectively, and C accounts for background [33,56]. The mean fluorescence lifetime is defined as $\tau_m = \alpha_1\tau_1 + \alpha_2\tau_2$. To enhance the fluorescence counts in each decay, a 3×3 bin comprising nine neighboring pixels was employed (>1000 photons/pixel [57]). Whole-cell masks were generated using Cellpose (model “cyto2”) and manually corrected as needed [58,59]. Masks were binarized, and FLIM data were extracted from all cells in a single FOV using a custom Python library, “cell analysis tools” [60]. Plots and statistics were generated using GraphPad Prism 10.

5.3.6 Widefield ORI and Analysis

Widefield ORI was performed using a Nikon Ti-2E coupled to a SOLA light engine (380 to 660 nm, Lumencor, Beaverton, Oregon, United States). All data were collected with NIS Elements software (Nikon) using a $10\times$ air, 0.45 NA objective (Plan Apo, Nikon), and Hamamatsu Orca-Flash digital CMOS camera. NAD(P)H was excited for 300 to 500 ms through a 360/40 nm filter at 50% power (0.0011 mW/mm^2), and emissions were collected using a 400 nm dichroic mirror and a standard 4',6-diamidino-2-phenylindole (DAPI, 460/50 nm) filter. FAD was sequentially excited for 1.3 to 1.7 s through a 480/30 nm filter at 50% power (0.0019 mW/mm^2) and emissions collected using a 505 nm dichroic mirror and a standard fluorescein isothiocyanate (FITC, 535/20 nm) filter. A single FOV (image size $\frac{1}{4}$ $1.33 \text{ mm} \times 1.28 \text{ mm}$; 2044×2048 pixels) was taken of each gel, with three replicates per gel formulation. Images were acquired on days 6, 8, 10, and 16 post-

differentiation (differentiation); on days 6, 8, 10, and every 3 days thereafter through day 30 (early maturation); or on days 6, 8, 10, 16, 23, 30, and every 10 days thereafter (late maturation).

NAD(P)H and FAD widefield autofluorescence images were analyzed using Fiji [61]. A rolling ball subtraction was performed to correct vignetting on the NAD(P)H fluorescence intensity image. The resulting image was thresholded to separate cells from the background signal (e.g., substrate autofluorescence) and binarized to create a mask, where background pixels are assigned a value of 0 and pixels within cells a value of 1 (Figure 5.1). This mask was multiplied by both the raw NAD(P)H and FAD intensity images, and the mean fluorescence intensity of the masked cell pixels was measured from each channel. We expect day-to-day variation in the wide field system (e.g., minor drift, variation in exposure time), and illumination through an auto-fluorescent substrate may affect intensity-based measurements. To account for these factors, we measured the average fluorescence intensity from three background (cell-free) locations within each gel. We calculated a background-normalized redox ratio for all cells on each gel as follows (summarized in Figure 5.1):

$$I_{\text{NAD(P)H}} = \frac{I_{\text{cell NAD(P)H}}}{I_{\text{background NAD(P)H}}},$$

$$I_{\text{FAD}} = \frac{I_{\text{cell FAD}}}{I_{\text{background FAD}}},$$

$$\text{Normalized ORR} = \frac{I_{\text{NA(P)H}}}{I_{\text{NAD(P)H}} + I_{\text{FAD}}}.$$

All plots and statistics were generated using GraphPad Prism 10.

5.4 Results

5.4.1 NAD(P)H Protein Binding Increases during iPSC-CM Differentiation on Synthetic Hydrogels

CMs undergo extensive metabolic changes during development, exhibiting an early reliance on glycolysis and shifting primarily to fatty acid oxidation during terminal differentiation [54]. Upregulation of oxidative phosphorylation increases NADH protein binding, which can be detected non-invasively using autofluorescence FLIM [33,40]. To date, autofluorescence FLIM has not been used to study metabolic changes in iPSC-CMs throughout the full differentiation and >30 day maturation processes. Furthermore, repeated label-free metabolic imaging has not been performed on iPSC-CMs undergoing these processes on synthetic substrates. Multiphoton NAD(P)H FLIM was therefore first used to characterize expected metabolic shifts and complement later widefield redox analysis. Imaging was performed at the CPC stage (day 6) through differentiation (day 16). Twenty-seven synthetic hydrogel formations and coated plates, Matrigel, and Geltrex were seeded from the same batch of CPCs (Supplementary Table 5.2). The imaged cells were fixed on day 16, and flow cytometry was performed to verify differentiation efficiency (Figure 5.2A). Most cells exhibited high (>60%) levels of cTnT expression, regardless of substrate. We observed an increase in NAD(P)H mean lifetime (τ_m) and the protein-bound fraction (α_2) by day 10 relative to days 6 and 8, with further increases by day 16 (Figure 5.2B-C). This indicates that cellular metabolism shifts to a more oxidative phenotype during differentiation and confirms the expected changes in cells differentiated on synthetic substrates.

5.4.2 Widefield ORI Maintains Viability and Purity of iPSC-CMs

Multiphoton NAD(P)H FLIM is sensitive to the local molecular environment and can achieve high spatial resolution. However, acquiring and analyzing multiphoton FLIM images are time consuming, requiring expensive hardware and specialized training. As a result, we next tested whether single-photon ORI with a commercial epifluorescent widefield microscope provides similar sensitivity to screen iPSC-CM differentiation and maturation conditions. First, to ensure UV light exposure did not impact differentiation, CPCs were plated on 12 representative gel formulations (plate coating, Matrigel, Geltrex, 9 synthetics; Supplementary Table 5.3) each with three replicates (36 gels total). Two experimental gel sets were generated in the same 96-well angiogenesis plate, and one set was imaged on days 6, 8, 10, and 16 using ORI. The other set was subjected to the same environmental and media changes (e.g., moving between incubators) but not directly exposed to UV light. Flow cytometry was performed on day 16 to assess differentiation outcome (% cTnT and cell number). No significant differences ($p > 0.05$) were observed between the UV-exposed and non-exposed groups (Figure 5.3).

5.4.3 Widefield ORR Decreases during iPSC-CM Differentiation on Synthetic Hydrogels

We next investigated if the expected metabolic shift away from glycolysis could be observed using widefield ORI performed using a commercial microscope and free analysis software. Twelve gel conditions were generated in triplicate (Supplementary Table 5.3), and CPCs were seeded 5 days post-differentiation. ORR was calculated from widefield fluorescence intensity images on days 6, 8, 10, and 16 post-differentiation

before cells were fixed for flow cytometry in three separate screens (the average for all three screens was $\geq 50\%$ cTnT for each gel type). ORR initially increases in the CPC stage (6 to 10) and then decreases by the differentiation timepoint (day 16; Figure 5.4).

5.4.4 Widefield ORR Can Monitor iPSC-CM Metabolic Maturation on Synthetic Hydrogels

We next investigated if cell metabolism could be monitored in maturing iPSC-CMs using wide field ORI. Eighteen different synthetic gel formulations plus plate coatings, Matrigel, and Geltrex (Supplementary Table 5.4) were made in triplicate and seeded with high differentiation efficiency cells ($>70\%$ of cells become CMs in prior experiments from the same batch, confirmed by flow cytometry) [3]. ORR was measured at days 6, 8, and 10 and then every 3 days thereafter until early maturation (day 30). We found that ORR calculated from widefield fluorescence images was sensitive to metabolic changes occurring post-differentiation, and by day 30, ORR of cells seeded on all gel formulations significantly decreased relative to day 16 (Figure 5.5A). Long-term culture (beyond early maturation) is known to enhance iPSC-CM maturation, resulting in increased cell size, alignment, and maturation-related gene and protein expression [15,62]. We performed extended culture and widefield imaging up to 100 days post differentiation to observe if ORI is sensitive to further metabolic changes that occur across this timescale. We found that iPSC-CM ORR continued to decrease beyond the early maturation timepoint (day 30) and reached its lowest measured point at 100 days, indicating further increases in oxidative metabolism throughout extended culture (Figure 5.5B). This trend was most

notable in the synthetic hydrogels, with significant decreases observed between the early (day 30) and late (days 90 to 100) stages (Supplementary Figure 5.1).

5.4.5 Widefield ORR Distinguishes Between High and Low Differentiation

Efficiency of Batches in the Cardiac Progenitor Stage

Confirming batch purity of stem cell-derived CMs is often labor-intensive, requiring techniques such as flow cytometry or qPCR that are typically performed two weeks after initializing differentiation [3,8,9,63]. Label-free FLIM of metabolic coenzymes can separate stem cell-derived CMs by differentiation efficiency within 24 hours of initializing differentiation [46]. We investigated if widefield ORI provides sufficient sensitivity to separate cell batches by differentiation efficiency in the CPC stage. Seventeen gel formulations (Supplementary Table 5.6) were generated in triplicate and duplicated in a second plate. One plate was seeded with high efficiency cells (>70% CM population expected) and the second with low-efficiency cells (<35% CM population expected). Plates were imaged in the same session on days 6,8, and 10 and then every 3 days thereafter until day 30. Cells were fixed on day 30, and flow cytometry for an early structural marker of CM maturation (MLC2) [64,65] was performed as an independent measure of maturity.

In the early CPC stage (day 6), low-efficiency cells had a significantly lower ORR compared with high-efficiency cells, and this difference persisted beyond differentiation (day 16; Figure 5.6A). As cells began to mature, the distinction between the groups was lost (day 30). High-efficiency cells exhibited greater decreases in ORR throughout maturation (−8.15% average ORR day 6 versus 30) compared with low-efficiency cells

(-5.09% average ORR day 6 versus 30; Figure 5.6A). On day 30, the higher-efficiency cells also exhibited higher expression levels of the ventricular isoform of the myosin light chain (MLC2v, Figure 5.6B), one structural indicator of CM maturity, whereas the low-efficiency cells expressed more of the atrial and intermediate forms (MLC2a, av: Figure 5.6B). These data support a more mature phenotype in the high-efficiency cells on day 30, with lower ORR indicating a shift toward oxidative metabolism and greater metabolic maturity and higher MLC2v expression indicating more structural maturity.

5.5 Discussion

Here, we report a widefield optical imaging method to monitor metabolic changes in iPSC differentiation and subsequent CM maturation. We used NAD(P)H multiphoton FLIM to confirm an oxidative shift in iPSCs undergoing differentiation on PEG-based hydrogels. We used widefield ORI to decrease imaging time and complexity and observed a decrease in ORR, indicating more oxidative metabolism, with differentiation and maturation for cells on multiple hydrogel formulations. We demonstrated extended culture on synthetic hydrogels and repeatedly performed widefield ORI up to 100 days post-differentiation and observed redox changes throughout maturation. Finally, widefield ORI distinguished differentiation efficiencies between CPCs as early as 6 days post-differentiation, with higher-efficiency cells exhibiting both a greater decrease in ORR and increased structural maturity (%MLC2v) by day 30 compared with lower-efficiency cells.

To date, label-free metabolic imaging has not been performed in iPSC-CMs differentiating and/or maturing on synthetic substrates. Therefore, both widefield ORI and NAD(P)H FLIM were performed to provide a comprehensive and complementary

characterization of autofluorescence in these samples, which have not been previously imaged using autofluorescence FLIM or intensity methods. For the imaging parameters used in this study, widefield ORI reduces imaging and analysis time approximately fourfold compared with multiphoton autofluorescence FLIM. Multiphoton FLIM provides subcellular resolution, optical sectioning, and is sensitive to changes in the molecular state and environment. While both FLIM and widefield redox imaging are both sensitive to redox pool changes, they have differing specificities [34]. As a result, we do not necessarily expect a one-to-one correspondence between our NAD(P)H FLIM and ORI data [34,40,66–68]. However, a widefield imaging configuration presents certain advantages such as lower cost, wide availability, and suitability for rapid screening of multiple culture conditions as demonstrated here. Since widefield ORI does not perform optical sectioning, signals are integrated over $\sim 100\ \mu\text{m}$, which may be advantageous for rapidly sampling multiple depths within 3D samples. Compared with single-cell segmentation and analysis, FOV-level analysis also greatly decreases computational time, which could enable real-time monitoring during biomanufacturing.

Intensity-based measurements such as ORI generally need to be normalized to account for day-to-day system variability and changes in background effects. Here, we note that our synthetic hydrogels are weakly autofluorescent in the DAPI and FITC channels, potentially due to both material properties and the sequestration of cell-secreted factors over time (Figure 5.2). This background signal may shift over time and likely contributes a small amount to the overall signal. We accounted for daily system variations and background contribution by normalizing the cell autofluorescence to the

fluorescence intensity of the cell-free gel (background) in both channels before calculating ORR. This normalization was performed on an individual gel level, accounting for any differences that may occur in focus or exposure between gels. For comparison, we also included ORR measured from cells on coated plates at each timepoint to assess the metabolism of cells on novel hydrogels relative to a biological standard. We note that a standard fluorophore (e.g., rhodamine) or day-matched cell-free gel could also be used to account for the background variations and contribution.

In this study, we seeded all gels with cells from the same initial cardiac progenitors so that metabolic changes can be attributed to differences in substrate composition. The protocol is expected to produce a relatively pure CM population [3] and observed metabolic changes dominated by the differentiation process in iPSC-CMs [46] rather than proliferation or senescence [69–71]. We found that ORI can detect increased oxidative activity during early maturation (decreased ORR over time) and separates cells by differentiation efficiency. Furthermore, we demonstrated the ability to perform extended culture and repeated ORI on synthetic hydrogels. ORR continued to decrease beyond the early maturation (day 30) stages, indicating continued metabolic shifts over 100 days. We note that this trend is more robust in cells seeded on synthetic hydrogels (Figure 5.5B, magenta) relative to Matrigel or Geltrex (gray): significant decreases in ORR continue in synthetic gels alone from early to late maturation (Supplementary Figure 5.1). These differences may be due to known difficulties with extended culture on Matrigel, as it tends to degrade in an uncontrollable manner over time [25].

The hydrogel conditions tested here were selected both for their relevance to the cardiac environment and their ability to support CPC adhesion and survival. This considerably limited the number of substrate conditions that were able to be tested in these experiments. This may have contributed to the lack of sensitivity of widefield ORI to substrate-induced metabolic changes. We found that widefield ORI is not sensitive to metabolic changes induced by substrate differences studied here (e.g., stiffness, concentration of primary and/or secondary peptide).

Currently, standard methods to determine differentiation efficiency in stem cell batches or maturation of the resulting cell types are labor-intensive and destructive, confining these techniques to a single timepoint. By contrast, our workflow is medium throughput and uses a standard commercial widefield system that is commonly found in biological laboratories and free-to-use software (ImageJ/Fiji), which could be widely adopted by academic and industrial groups that do not have significant optics experience. Future improvements to this workflow will include streamlining the background normalization steps to further reduce computational time. While widefield ORI cannot distinguish between specific metabolic pathways since it relies on whole cell NAD(P)H and FAD signals, the touch-free nature of this method enables repeated measurements at any point (stem cell culture through maturation) and can be combined with standard metabolic perturbations [46] Furthermore, widefield ORI was not found to affect iPSC-CM viability or purity (Figure 5.3), and all flow cytometry was performed on imaged cells. This demonstrates that the imaged cells can later be used for complementary endpoint

analyses in the future such as immunostaining, quantitative proteomics or metabolomics [72], and single-cell RNA sequencing [73,74].

5.6 Conclusion

We demonstrated a label-free widefield imaging technique to monitor the metabolic phenotype of iPSC-CMs differentiating and maturing on synthetic hydrogels. This method is medium throughput and uses accessible microscope modalities and analysis tools. Multiphoton FLIM confirmed expected metabolic changes in iPSC-CMs during maturation on hydrogels. We demonstrated using widefield ORI that glycolytic metabolism decreases in differentiated cells during early and late maturation in iPSC-CMs cultured on synthetic hydrogels, and the same cells can be repeatedly monitored and used for complementary analyses. Furthermore, widefield ORI distinguishes between high and low differentiation efficiency batches in the early CPC stage. Overall, these tools provide an accessible, medium throughput method to monitor iPSC metabolism in response to interventions that aim to increase batch yield, consistency, and maturity without manipulating or destroying the cells.

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5.9 Figures

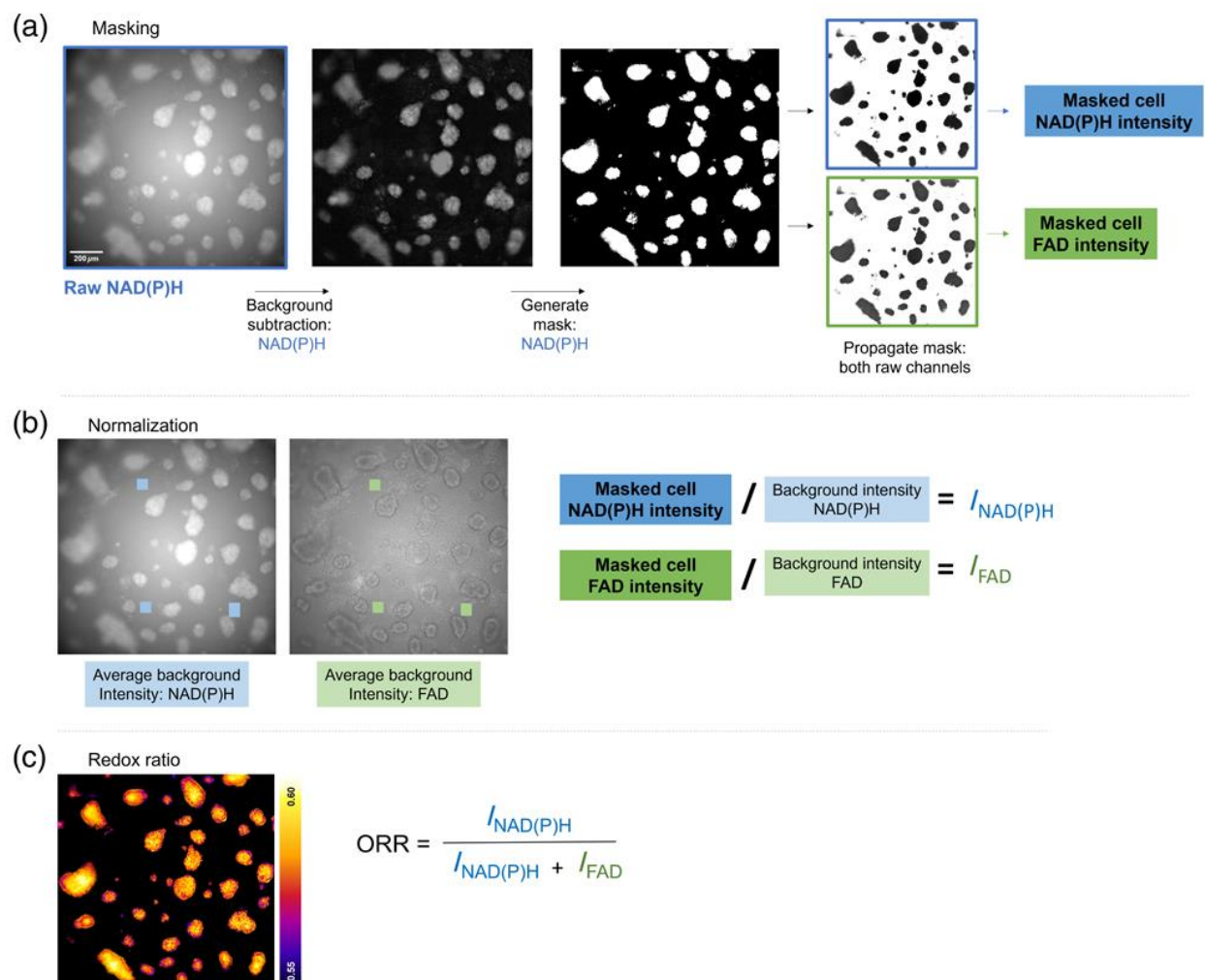


Figure 5.1. Widefield optical redox analysis workflow.

(A) A rolling ball subtraction was performed on raw NAD(P)H images prior to thresholding, generating binary masks separating cell autofluorescence from the background. This mask was applied to both raw image channels to measure cellular NAD(P)H and FAD in each FOV. **(B)** The average intensity of three cell-free areas was measured to account for gel autofluorescence. Cellular NAD(P)H and FAD signal were normalized to background intensity on a per-gel level. **(C)** Background-normalized NAD(P)H and FAD intensity values were used to calculate ORR per FOV. Scale bar = 200 μm .

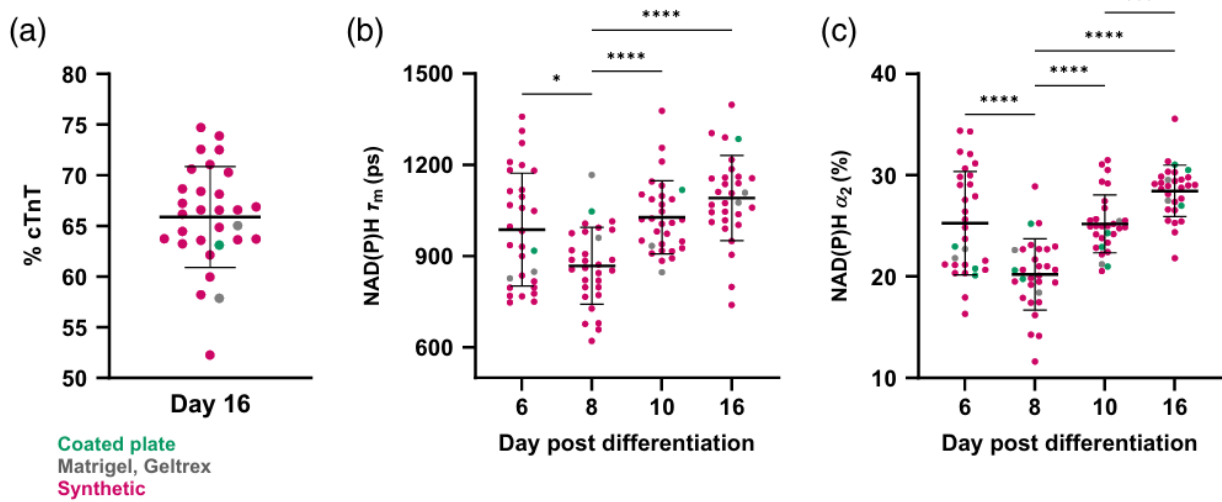


Figure 5.2. NAD(P)H protein binding increases during iPSC-CM differentiation on synthetic hydrogels.

CPCs were seeded on synthetic hydrogels (pink, 27 formulations), coated plates (green), and Matrigel or Geltrex (gray) with three gel replicates per condition. **(A)** Differentiation was confirmed using flowcytometry for cTnT expression (%cTnT). **(B)** Average NAD(P)H lifetime (τ_m) and **(C)** protein-bound fraction (α_2) were measured per gel and shown to increase throughout differentiation, indicating more oxidative metabolism. Repeated measures analysis of variance (ANOVA) with Tukey's multiple comparisons test, * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$. Each dot = cells from three replicate gels.

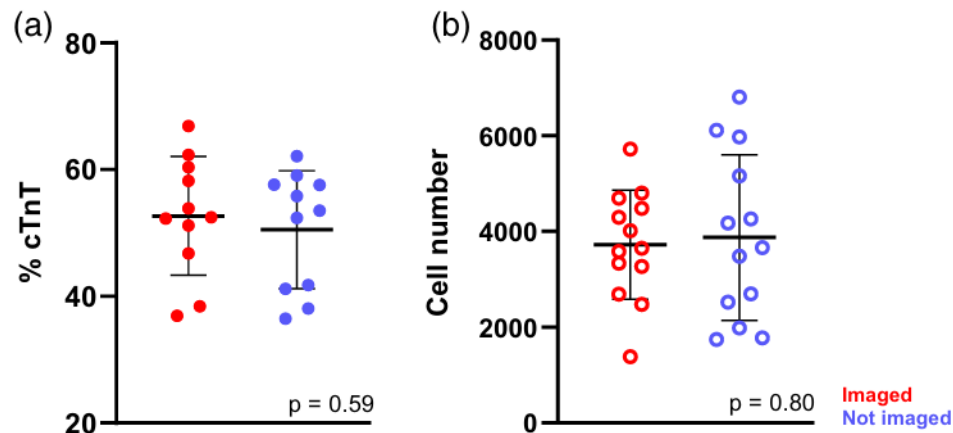


Figure 5.3. Widefield redox imaging with UV excitation is minimally phototoxic to iPSC-CMs.

Twelve representative gel formulations were seeded with CPCs and imaged on days 6, 8, 10, and 16 post differentiation. A matched set of cells in the same plate were not imaged. **(A)** Flow cytometry for differentiation markers (%cTnT) and **(B)** cell number on day 16 revealed no significant differences between the imaged and non-imaged groups, indicating minimal phototoxic effects of ORI during differentiation. Unpaired t-test. Each dot $\frac{1}{4}$ cells from three replicate gels.

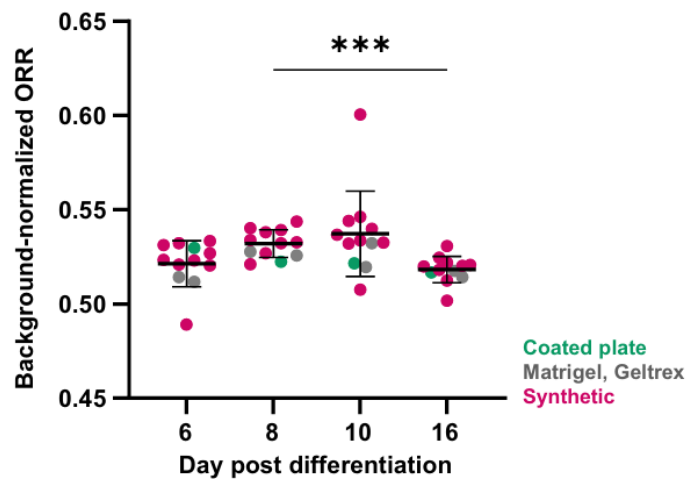


Figure 5.4. Widefield ORR shifts during iPSC-CM differentiation on synthetic hydrogels.

CPCs were seeded on synthetic hydrogels (nine formulations), coated plates, Matrigel, or Geltrex, with three gel replicates per screen. ORR initially increases and then begins to decrease by the differentiation timepoint, indicative of decreasing glycolytic activity.

Repeated measures ANOVA with Tukey's multiple comparisons test, *** $p < 0.001$. Each dot = cells from nine replicate gels (three per screen).

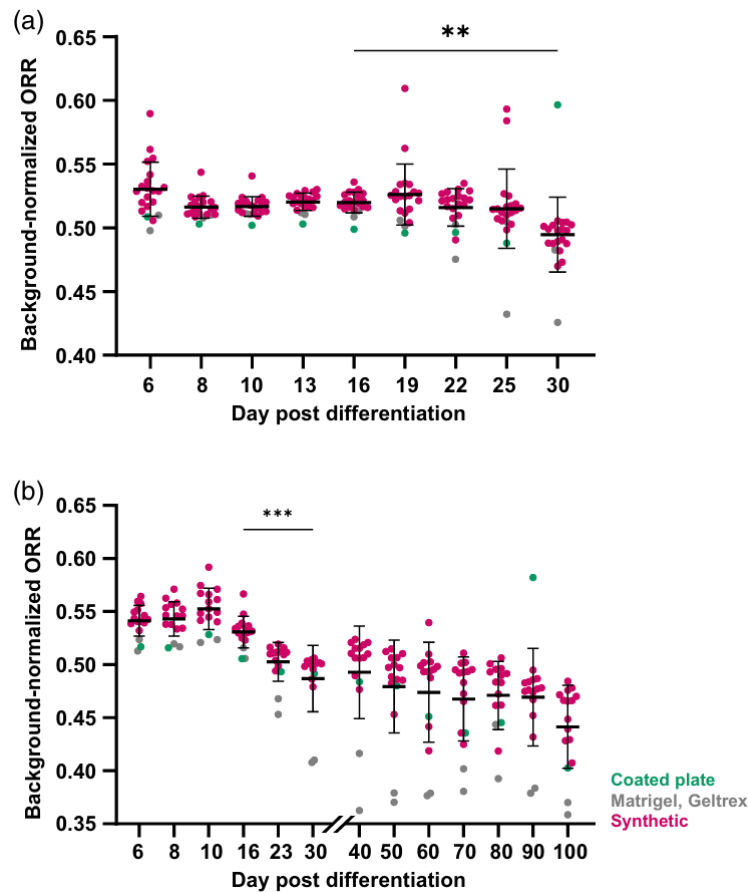


Figure 5.5. Widefield ORR becomes more oxidized during early and late iPSC-CM maturation.

CPCs were seeded on synthetic hydrogels, coated plates, Matrigel, or Geltrex with three gel replicates per formulation. **(A)** ORR was measured at multiple timepoints through early (up to day 30) or late maturation (day 30+). ORR significantly decreased relative to ORR at the differentiation timepoint (day 16 versus 30). **(B)** ORR continued declining during late maturation (days 30 to 100), with cells on synthetic hydrogels showing further decreases out to day 100. Brown–Forsythe and Welch ANOVA with Dunnett's T3 multiple comparisons test, ** $p < 0.01$, *** $p < 0.001$. Each dot = cells from three replicate gels.

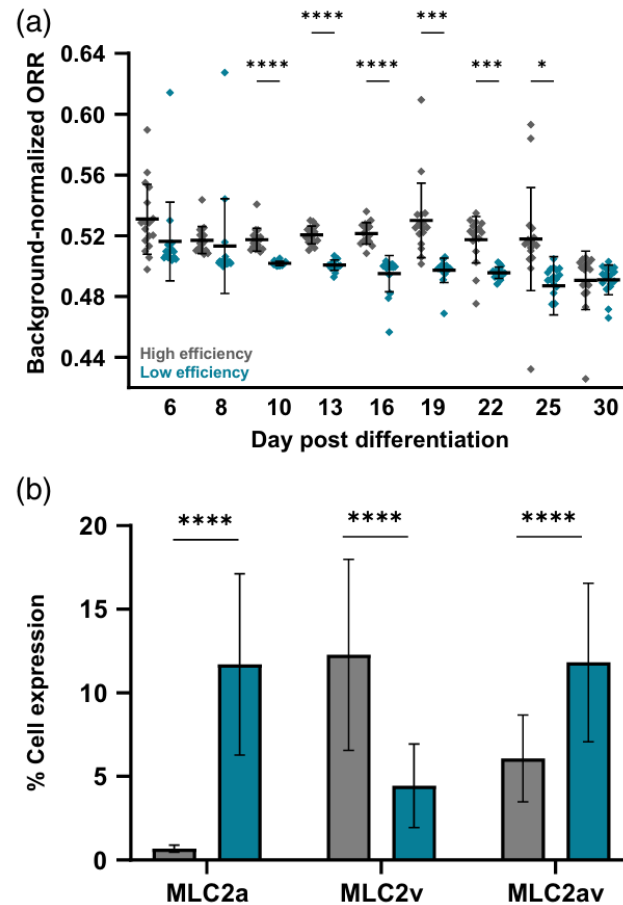


Figure 5.6. Widefield ORI separates high and low differentiation efficiency groups at the cardiac progenitor stage.

High- and low-efficiency CPCs were seeded on identical gels, and widefield ORI was measured through early maturation. **(A)** ORI significantly differs between efficiency groups at the CPC stage, with high-efficiency cells initially having a higher ORI. This distinction disappears by early maturation (day 30). High-efficiency cells exhibit a lower ORI and more oxidative phenotype by day 30 relative to day 6. **(B)** High-efficiency cells express greater levels of the ventricular isoform of MLC2, indicating increased structural maturity relative to their low-efficiency counterparts. Welch's t-test, **** $p < 0.0001$. Brown–Forsythe and Welch ANOVA with Dunnett's T3 multiple comparisons test, * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$. Each dot = cells from three replicate gels.

5.10 Supplementary Figures

Peptide	Primary/secondary	Abbreviation	Derived from
Linear RGD (arginylglycylaspartic acid)	1°	L	Fibronectin
Cyclic RGD (arginylglycylaspartic acid)	1°	C	Fibronectin
IKVAV (Ile-Lys-Val-Ala-Val)	2°	I	Laminin
YIGSR (Tyr-Ile-Gly-Ser-Arg)	2°	Y	Laminin

Supplementary Table 5.1 General synthetic gel information.

Concentration (mM)	1°	Concentration (mM)	2°	Stiffness (kPa)
7	L	-	-	0.5
7	L	1.5	I	0.5
3.5	C	-	-	0.5
3.5	C	1.5	Y	0.5
7	C	3	I	0.5
7	C	3	Y	0.5
3.5	L	-	-	2
7	L	-	-	2
7	L	1.5	I	2
3.5	C	-	-	2
7	C	-	-	2
7	C	3	Y	2
3.5	L	-	-	4
3.5	L	3	Y	4
3.5	C	-	-	4
3.5	C	3	I	4
3.5	C	3	Y	4
7	C	3	Y	4
7	L	1.5	I	6
7	L	3	Y	6
3.5	C	1.5	Y	6
3.5	C	3	Y	6
7	C	1.5	I	6
7	C	3	Y	6
7	C	-	-	10
7	C	1.5	I	10
7	C	3	I	10
Matrigel		Gel format		< 1 ²⁵
Geltrex		Gel format		< 1 ²⁵
Matrigel		Coated plate		-

Supplementary Table 5.2 FLIM differentiation formulations. (Figure 5.2)

Concentration (mM)	1°	Concentration (mM)	2°	Stiffness (kPa)
7	L	-	-	2
3.5	C	-	-	2
7	C	3	Y	2
7	L	1.5	I	6
3.5	C	3	Y	6
7	C	1.5	I	6
7	C	-	-	10
7	C	1.5	I	10
7	C	3	I	10
Matrigel		Gel format		< 1
Geltrex		Gel format		< 1
Matrigel		Coated plate		-

Supplementary Table 5.3 ORI differentiation formulations. (Figure 5.3-5.4)

Concentration (mM)	1°	Concentration (mM)	2°	Stiffness (kPa)
7	L	1.5	I	0.5
3.5	C	-	-	0.5
3.5	C	1.5	Y	0.5
7	L	-	-	2
7	L	1.5	I	2
3.5	C	-	-	2
7	C	3	Y	2
3.5	L	-	-	4
3.5	C	-	-	4
3.5	C	3	I	4
7	C	3	Y	4
7	L	1.5	I	6
7	L	3	Y	6
3.5	C	3	Y	6
7	C	1.5	I	6
7	C	-	-	10
7	C	1.5	I	10
7	C	3	I	10
Matrigel		Gel format		< 1
Geltrex		Gel format		< 1
Matrigel		Coated plate		-

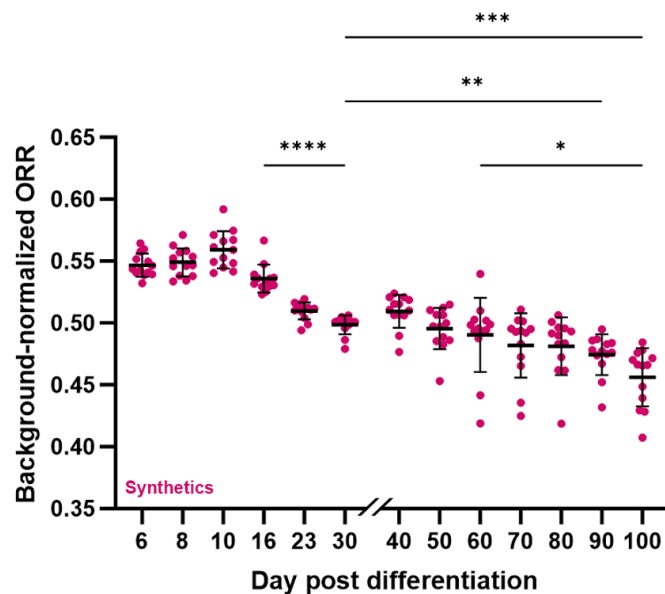
Supplementary Table 5.4 Maturation formulations. (Figure 5.5A)

Concentration (mM)	1°	Concentration (mM)	2°	Stiffness (kPa)
3.5	C	-	-	0.5
3.5	C	3	Y	0.5
7	L	-	-	2
3.5	C	-	-	2
3.5	C	1.5	Y	2
7	C	3	Y	2
7	C	3	I	2
7	L	1.5	I	6
3.5	C	3	Y	6
7	C	1.5	I	6
7	C	-	-	10
7	C	1.5	I	10
7	C	3	I	10
Matrigel		Gel format		< 1
Geltrex		Gel format		< 1
Matrigel		Coated plate		-

Supplementary Table 5.5 Maturation formulations. (Figure 5.5B)

Concentration (mM)	1°	Concentration (mM)	2°	Stiffness (kPa)
7	L	1.5	I	0.5
3.5	C	1.5	Y	0.5
7	L	-	-	2
7	C	3	Y	2
3.5	L	-	-	4
3.5	C	-	-	4
3.5	C	3	I	4
7	C	3	Y	4
7	L	1.5	I	6
7	L	3	Y	6
3.5	C	3	Y	6
7	C	1.5	I	6
7	C	-	-	10
7	C	1.5	I	10
7	C	3	I	10
Matrigel		Gel format		< 1
Geltrex		Gel format		< 1

Supplementary Table 5.6 High vs. low formulations. (Figure 5.6)



Supplementary Figure 5.1 Widefield ORR becomes more oxidized in cells on synthetic hydrogels throughout late iPSC-CM maturation.

CPCs were seeded on synthetic hydrogels at the progenitor stage and repeatedly imaged through the differentiation (day 16), early maturation (day 30), and late maturation (>day 30) timepoints. ORR significantly declines beyond the early maturation timepoint, with continued decreases observed through day 100.

Chapter 6 : Conclusions and Future Directions

6.1 Conclusions

The research described in this thesis demonstrated the use of a multi-variate, multi-screen approach to study the effect of biomaterials on cell behavior. In the cardiovascular field especially, there is a crucial unmet need for novel therapies as cardiovascular diseases represent the leading cause of death in the world [1,2] and yet discovery of novel therapies remains limited [3]. Stem cells bear an enormous therapeutic potential which remains unfulfilled notably due to heterogeneous differentiations [4,5], lack of scalability [6-8], and limited maturation [9]. The cell's microenvironment is increasingly shown to play a considerable part in cell behavior [10-12].

To manipulate the cell microenvironment as desired, Chapter 2 focused on varying poly(ethylene glycol) (PEG) hydrogel stiffness and adhesion ligand type and concentration for adhesion and differentiation of iPSC-derived cardiac progenitor cells (iPSC-CPCs) into iPSC-derived cardiomyocytes (iPSC-CMs). The use of PEG hydrogels resulted in a greater than 20 % increase in iPSC-CM differentiation efficiency as well as increased reproducibility of differentiation efficiencies both across experiments and across replicates, indicating a significant improvement in iPSC-CM yield. These results highlight the clear potential of the PEG hydrogel platform to serve as the cell culture substrate in iPSC-CM manufacturing.

Due to the significant advantages of using PEG hydrogels for iPSC-CM differentiation, we further assessed the influence of PEG hydrogels on iPSC-CM maturation in Chapter 3. By conducting a multi-variate multi-screen study, two distinct

PEG hydrogel formulations were identified which enhance iPSC-CM maturation. In contrast with our previous differentiation studies, here, the PEG hydrogel platform had an overall similar influence on iPSC-CM maturation as the traditional cell culture substrate Matrigel. Two PEG hydrogel formulations, both demonstrating low stiffnesses and a cRGDfC peptide, resulted in iPSC-CMs demonstrating greater overall maturation, as assessed by studying structural, metabolic, and functional maturation metrics. The application of an optimization modeling tool significantly improved the multi-variate screening approach by identifying PEG hydrogel formulations that supported greater iPSC-CM maturation. The multi-variate, multi-screen optimization study developed here could be generalized and applied to identify optimized biomaterials for other targeted cell behavior.

In Chapter 4, we studied the influence of an additional material variable, namely the PEG hydrogel's surface topography, on iPSC-CM behavior. The PEG hydrogel platform was highly amenable to modification of surface topography according to our desired pattern, highlighting an additional advantage of this platform, especially in comparison to the traditional Matrigel substrate which was not amenable to topography modification using this patterning approach. Our surface topography was seen to direct cell elongation and aggregate alignment of iPSC-CPCs over a short period of time, eventually resulting in long-term iPSC-CM myofibril and nuclear alignment. These results indicate the importance of biomaterials in cell culture as even short-term effects can yield long-term changes in cell behavior.

Finally, Chapter 5 assessed the incorporation of a label-free optical redox imaging workflow into the cardiomyocyte differentiation process on PEG hydrogels for real-time analysis of cell behavior. Label-free imaging confirmed the metabolic changes in iPSC-CMs during differentiation and maturation on PEG hydrogels and successfully identified high differentiation efficiency cell populations at the cardiac progenitor stage. This approach supported live cell analysis and confirmed expected metabolic changes in iPSC-CM during development and could therefore be applied to monitor and assess iPSC-CM manufacturing.

6.2 Ongoing Work and Future Directions

The research described in this dissertation contributes to a growing understanding of the importance of biomaterials on cell behavior during *in vitro* culture, but continued efforts are necessary to fully elucidate the role of microenvironmental determinants on cell behavior. Biomaterials have applications in a broad range of *in vitro* and *in vivo* studies for tissue engineering or regenerative medicine studies. Some ongoing and future avenues for these areas of research are presented here.

Chapter 2 highlighted the direct advantages of using a fully synthetic PEG hydrogel platform as we observed immediate increases in iPSC-CM differentiation efficiency and reproducibility of the differentiations. Future work would look at the translatability of these advantages to cell lines other than WTC11 used in the studies presented here. Stem cell behavior changes with respect to cell lines [13,14] and to account for this, cardiomyocyte differentiation protocols are modified according to the stem cell line [15,16]. The use of the PEG hydrogel platform described here may demonstrate greater reproducibility

across stem cell lines as well. Further, the optimization modeling approach established in Chapter 3 demonstrated rapid identification of high potential PEG hydrogel formulations for iPSC-CM maturation within a relatively large parameter space. Growing interest has resulted in the development of modeling tools for improved robustness of high throughput screening of biomaterials for cell culture [17]. The optimization approach described here could be generalized to identify biomaterial properties that support other cell behavior of interest. Additionally, ongoing work in iPSC-CM maturation in Chapter 3 is looking at the compounded effect of greater maturation of iPSC-CMs cultured on PEG hydrogels over a longer period of time. Studies show increased iPSC-CM maturation in long term culture [18]. With increased iPSC-CM maturation observed on a relatively short timeframe on our hit PEG hydrogels, iPSC-CM maturation may be considerably greater on our hit PEG hydrogels after a longer period of time in culture.

An increasing number of studies are developing fine-tuned culture conditions optimized for specific cell behavior. To look at a significantly greater number of microenvironmental variables, studies are incorporating dimensionality reduction tools along with machine learning algorithms to rapidly identify culture conditions [17,19-22] which could be applied in the context of identifying combinations of microenvironmental properties that yield a desired cell behavior, as explored in part here. The screening approach described in Chapter 3 could also be applied to a larger parameter space (greater number of material variables, greater range for each variable). This approach could then be combined with an initial dimensionality reduction step to simplify the experimental workflow and especially the number of samples and screens required to

identify the targeted maximum of the defined parameter space. Continued work studying the interactions of various material variables on cell behavior would result in a greater understanding of these relationships to then be applied in the development of a defined material with respect to the target cell behavior.

6.3 References

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