3D Human Induced Pluripotent Stem Cell-derived Bioengineered Skeletal Muscles for Tissue, Disease and Therapy Modelling

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Abstract

Skeletal muscle is a complex tissue composed of multinucleated myofibres responsible for force generation, supported by multiple cell types. Many severe and lethal disorders affect skeletal muscle; therefore, engineering models to reproduce such cellular complexity and function is instrumental for investigating muscle pathophysiology and developing therapies. Here, we detail the modular 3D bioengineering of multilineage skeletal muscles from human induced pluripotent stem cells, which are first differentiated into myogenic, neural and vascular progenitor cells, and then combined within 3D hydrogels under tension to generate an aligned myofibre scaffold containing vascular networks and motor neurons. 3D bioengineered muscles recapitulate morphological and functional features of human skeletal muscle, including establishment of a pool of cells expressing muscle stem cell markers. Importantly, bioengineered muscles provide a high-fidelity platform to study muscle pathology, such as emergence of dysmorphic nuclei in muscular dystrophies caused by mutant lamins. The protocol is easy to follow for operators with cell culture experience and takes between 9 and 30 days, depending on the number of cell lineages in the construct. We also provide examples of applications of this advanced platform for testing gene and cell therapies *in vitro*, as well as for *in vivo* studies, providing proof-of-principle of its potential as a tool to develop next-generation neuromuscular or musculoskeletal therapies.

Introduction

1.1 Overview

Skeletal muscle is a complex tissue composed of contractile multinucleated myofibres. Upon damage, skeletal muscles regenerate myofibres through activation of resident stem cells named satellite cells, also known as muscle stem cells (MuSCs), which give rise to myoblasts, committed myogenic progenitors[3]. Several other cell lineages contribute to muscle function, homeostasis and regeneration, including motor neurons, endothelial, perivascular and immune cells[4,5]. Muscle repair and regeneration are impaired in muscle disorders such as muscular dystrophies[6]: severe, progressive and incurable genetic myopathies caused by mutations in genes involved in many aspects of muscle function, from sarcomeric contraction, to nuclear and membrane integrity. For decades, animal models and monolayer cell cultures have been the mainstay to study pathogenesis and develop experimental therapies for muscle disorders. However, their use is limited by ethical, species and microenvironment fidelity issues[7].

Human(ised) skeletal muscle models provide an advanced platform to study disease mechanisms, test therapies, and refine tissue replacement protocols. Similar strategies based upon organoid technology for other tissues/organs have delivered breakthroughs for precision medicine[8,9]. Although rodent cells have been engineered into artificial muscles[10–20], three-dimensional (3D) striated muscle platforms using human cells are still undergoing refinement [7,12,20–30]. Such human 3D muscle methodologies have, however, raised biological (e.g., inadequate proliferation and differentiation capacity), technical (e.g., invasive biopsies and poor cell availability) and ethical (e.g., human embryonic-cell derivation) concerns. To overcome such limitations, we and others have made three-dimensional artificial muscles using

human induced pluripotent stem cells (hiPSCs)[1,2,31–33]. Specifically, using healthy donor or muscular dystrophy hiPSCs we pioneered generation of 3D bioengineered and biocompatible hydrogels with aligned parallel muscle fibres (with associated PAX7+ cells[34]; figure 1-3), resembling healthy or diseased skeletal muscles[1]. Using this platform, we modelled the characteristic nuclear shape abnormalities of skeletal muscle laminopathies[1,2], which are incurable muscular dystrophies caused by dysfunctional nuclear envelope proteins (figure 4)[35]. We then generated more advanced, fully hiPSC-derived, isogenic multilineage 3D muscle models containing myofibres[36,37], vascular endothelial cells[38], pericytes[38], and spinal motor neurons[39,40] (figure 3)[1]. Moreover, we also achieved engraftment and vascularization *in vivo* of both single and multi-lineage artificial muscles upon implantation in immunodeficient mice (figure 5)[1].

Here we detail a modular protocol to obtain 3D artificial muscles using hiPSCs from healthy donors or from patients with muscular dystrophies, that can be mono- (muscle only), bi- (muscle + endothelial cells or motor neurons), tri- (muscle + endothelial cells + pericytes) or tetra-lineage (muscle + endothelial cells + pericytes + motor neurons). As a working example, we provide a stepwise guide of how to induce, image and quantify nuclear abnormalities in bioengineered muscles to model skeletal muscle laminopathies (figure 4). We also highlight proof-of-principle examples of testing gene therapy vectors *in vitro*, as well as generating and implanting 3D muscles for *in vivo* studies (figure 5).

1.2 Comparison with other methods

There are currently three main fully hiPSC-based platforms for advanced skeletal muscle modelling in a 3D, organoid-like setup[31,32,33]. Rao *et al.* generated inducible myogenic progenitor cells from healthy donor human embryonic stem cells and iPSCs capable of differentiating into bundles of skeletal myofibres by modulating Wnt signalling, followed by overexpression of the myogenic factor Pax7[31]. Although this method enables assessment of functional parameters in the bioengineered constructs, it has not been validated with a transgene-free, small molecule-based method of hiPSC differentiation capable of recapitulating developmental myogenesis, has not been extended to multilineage hiPSC differentiation and has not used hiPSCs from genetic muscle or neuromuscular diseases.

Most available systems to study and model neuromuscular junctions (NMJs) and diseases have been developed using murine cultures (examples include Cvetkovic *ell-derived*[19] and Machado *et al.*[41]). However, Osaki *et al.* combined motor neuron spheroids differentiated from hiPSCs of a patient affected by amyotrophic lateral sclerosis (ALS) with 3D hiPSC-derived skeletal muscle fibre bundles in an optogenetically controllable microfluidic device[32]. Similarly to Rao *et al.*, chemically or electrically induced muscle force contraction followed by Ca^{2+} transients were assessed in this platform. More

recently, a similar platform has been used by Paredes-Redondo *et al*. to model neuromuscular defects in Duchenne muscular dystrophy (DMD)[42]. Both systems require a specialised and complex microfluidic set up and have not assessed the effect mediated by addition of key cell types in the muscle constructs such as vascular cells; moreover, this setup has not been shown to be amenable for implantation *in vivo*.

Recently, the autoimmune neuromuscular disorder myasthenia gravis was modelled using hiPSC-derived organoids showing functional NMJs[33]. hiPSC-derived neuromesodermal progenitors generated 3D aggregates originating spinal cord neurons, skeletal muscle and Schwann cells capable of self-organising to form functional NMJs. This model relies on self-assembly of the cell populations and lacks external cues to enable systematic parallel alignment of myofibres.

Thus, although these platforms have their strengths (e.g., amenable for functional assessments, microfluidic device, organoid-like self-organisation), most have not been validated to model muscular dystrophies or lack the organisation of muscle structure and the higher order of complexity from the presence of other supporting and muscle interstitial cells.

1.3 Limitations of the platform

The platform we describe enables modelling phenotypic features of native muscle tissue. However, it currently exhibits suboptimal maturation, similarly to the vast majority of other hiPSC-derived 3D cultures. Nonetheless, we show here that this limitation does not impact on the potential of the platform to model severe muscle disorders with known congenital phenotypes. Further engineering the muscle constructs to include additional supporting cells (such as fibroblasts, fibro-adipogenic progenitors and/or macrophages)[5] and refining their organisation to prolong time in culture is likely to enhance maturation. More in-depth assessment of the PAX7+ population is also required to define their identity within the 3D muscle constructs (e.g., fetal myoblasts vs. satellite-like cells). Importantly, NMJs[43] form in our model[1], but have not yet been functionally assessed. Using bi-lineage 3D constructs containing optogenetically engineered motor neurons to induce muscle contraction could provide a strategy to test NMJ function and enhance maturation in future work.

From a translational perspective, the size of 3D muscle constructs can be scaled-down into microtissues to enhance suitability for screening programmes[44,45], or scaled-up with *in vitro* "vascularization" strategies[46]. Finally, the key matrix of our scaffold (fibrin) is biocompatible, readily available in clinical

grade formulations and amenable for autologous use[47], but minor components such as Matrigel require replacement with defined materials to facilitate scaling up production for pre-clinical applications.

1.4 Applications

This 3D artificial muscle platform can be used to model features of normal or diseased human skeletal muscle such as myofibre formation, alignment and growth or establishment of a stem cell pool and interaction with supporting cell types (e.g., vascular niche and NMJs; figure 3A). Importantly, with this platform we pioneered hiPSC-based 3D modelling of muscular dystrophy[2,1], unravelling key cellular phenotypic hallmarks of severe muscle disorders caused by defective nuclear envelope proteins. Specifically, 3D muscles outperformed conventional 2D cell monolayers when used to model skeletal muscle laminopathies, where they better reproduced the abnormally shaped nuclei associated with the underlying mutation in the nuclear envelope gene *LMNA*. Using this strategy, we defined nuclear major axis length in 3D as an objective phenotypic readout for screening programmes (figure 4). Nuclear dysmorphic features correlate with the severity of clinical presentation in a mutation-specific fashion, with the most common and severe *LMNA* R249W mutation[48] having the most deformed and elongated nuclei (figure 4F). Notably, this has been recently independently validated in an alternative 3D platform using *LMNA*-mutant myoblasts[49].

Phenotypic readouts obtained from this platform go beyond morphological parameters. We also measure key downstream functional skeletal muscle outcomes such as contractility and calcium dynamics (figure 3B, 3C; supplementary videos 1 and 2). These, and other readouts, can be used in therapy development pipelines, based upon small molecules or advanced products such as gene therapy vectors (examples in figure 5 A, B). The multilineage nature of our 3D constructs (figure 3A) enables testing cell specificity, toxicity and efficacy of drugs (new or repurposed) and advanced therapies (including viral vectors and cell therapies, figure 5A-C, supplementary video 3), in a humanised isogenic platform *in vitro*, opening new avenues in personalised medicine.

This 3D organoid-like platform has a strong impact for animal welfare in research, as it significantly refines, reduces and replaces animal use in pre-clinical research. The 3D, patient-specific, multicellular and isogenic nature of the artificial muscles reduces the number of animals required for *in vivo* work to validate a restricted number of disease pathogenesis or therapy/toxicity testing experiments. Of note, we have performed biocompatibility studies and shown engraftment of hiPSC-derived 3D muscles (both mono- or multi-lineage) in immunodeficient mice subjected to acute volumetric muscle injury/loss (figure 5C-H)[1].

We foresee this platform innovating and accelerating science and technology by: 1) studying human myogenesis to understand early disease phenotypes not captured by studies using biopsy-derived

skeletal myoblasts; 2) extending cellular multilineage components to less studied but critical niches and supporting cell types such as fibro-adipogenic progenitors and myotendinous junctions; 3) harnessing the potential of emerging microfabrication and microfluidic techniques to generate advanced muscle-on-chip models for precision medicine.

1.5 Experimental design

This protocol is modular in design, which allows for multiple variations and versatile applications. Its three main stages are: 1) monolayer expansion of progenitor cells to be used in making the muscles, followed by 2) generating and culturing the 3D artificial muscle constructs and finally 3) the use of engineered muscles in downstream applications (figure 1). Here we report using up to four different cell lineages (including the option of two different methods of progenitor cell derivation for two lineages). The choice of which progenitors to expand is based on the type of construct needed (figure 1B). Notably, we foresee that a number of alternative or new methods to derive myogenic and non-myogenic cell lineages can also be easily adapted to our protocol.

1.5.1 Preparation of cell lineages (steps 1-27)

This section gives a brief overview of the steps required to prepare the cell types required to generate a mono- or multi-lineage 3D muscle (with further details regarding their derivation in their associated publications). Standard practice is to first confirm the ability of the scaffold and biomaterial to support formation of aligned multinucleated skeletal myofibres, for example by using primary or immortalised adult human myoblasts, which are usually easier to source (even commercially) than hiPSC-derived myogenic cells (figure 2A-B). Next, we strongly advise quality-controlling the hiPSC starting populations for their identity, genomic stability, pluripotency and lack of mycoplasma contamination (figure 2B) prior to differentiation into derivatives. The derivatives should also be quality-controlled as per the original publications in which those specific protocols are detailed (figure 2C). Medium containing serum (such as foetal bovine serum; FBS) might give variable outcomes depending on the specific lot of serum and therefore we recommend batch-testing and stocking from lots/batches that give optimal results.

1.5.1.1 Skeletal myogenic cells (steps 1-6)

hiPSCs are a versatile source of skeletal myogenic progenitor cells for disease modelling, drug screening and tissue replacement studies, due to their controllable and extensive proliferation and differentiation capacity, minimal ethical concerns and non-invasive sampling[50]. Several protocols are available to generate hiPSC-derived skeletal myogenic cells[51]. Initial studies based upon controlled expression of myogenic regulators to obtain transplantable myogenic cells from hiPSCs[52–54] were followed by

transgene-based protocols to direct hiPSC differentiation into skeletal muscle[55–58]. State-of-the-art strategies now employ genomic-integration-free, small molecule-based protocols to generate myogenic cells by mimicking embryonic myogenesis[59–62].

In this protocol, four different combinations of cell lineages obtained from hiPSCs have been used to generate skeletal muscle constructs (figure 1). To derive skeletal muscle progenitors from hiPSCs, two approaches were used: a transgene-based and a transgene-free method.

Our transgene-based method relies on transiently expressing the myogenesis regulator MyoD via tamoxifen-induced nuclear translocation of a lentivirally-delivered MyoD-ER transgene in hiPSC-derived mesoangioblast-like cells to form hiPSC-derived inducible myogenic cells (HIDEMs)[56]. Cells should be thawed 24 hours in advance and incubated at 37 °C with 5% CO_2 and 3–5% O_2.

To recapitulate embryonic myogenesis with a transgene-free, small molecule-based method, we employed a commercially available kit to enhance standardisation[60] (Genea Biocells, now Myocea) in deriving a skeletal myoblast-like population termed 'iPSC-derived myogenic progenitors'. hiPSCs are passaged using Accutase® and the single cell suspension is seeded at 2.5 x 10^3 cells per cm^2 on Matrigel™-coated dishes and cultured in induction medium for 10 days at 37 °C with 5% CO_2 and 3–5% O_2. Upon completion of this first differentiation phase, cells are incubated at 37 °C with 5% CO_2 and 3–5% O_2 with myoblast medium for 8 days to obtain expandable and cryo-preservable hiPSC-derived myogenic progenitors.

Regardless of the differentiation method used, prior to use, it is essential to test the myogenic capacity of the generated hiPSC derivatives using a late differentiation marker such as the key sarcomeric component myosin heavy chain (MyHC), following a minimum of 5 days of differentiation.

1.5.1.2 hiPSC-derived motor neurons (steps 7-13)

Derivation of hiPSC-derived spinal motor neurons for integration into the 3D muscle construct can also be performed using two methods. The first is based upon seeding hiPSC-derived neurospheres[39] onto a polymerised artificial muscle where they attach and innervate the construct, mimicking native innervation. The second method[40] is based on co-seeding of single hiPSC-derived neural progenitor cells (NPCs) together with the other cell types within the artificial muscle, which increases the number of innervated muscle fibres. Both methods are extensively detailed in their respective publications[39,40] which contain important quality control steps, including confirming the identity of the neural progenitors (e.g., using markers such as Olig2 and differentiated motor neurons (such as using neurofilament protein SMI-32 and choline acetyltransferase (ChAT)).

1.5.1.3 Endothelial cells and pericyte-like cells (steps 14-27)

Endothelial cells (ECs) and pericyte-like cells (PCs) have been derived using another established protocol[63]. Here we start from step 15 in the aforementioned protocol and list the steps immediately prior to use of the cells in making tri- or tetra-lineage artificial skeletal muscles. FACS-enrichment was used to purify $CD31^+$ ECs from $CD31^-$ PCs, at variance with the magnetic bead purification method of the original paper[63]. We also outline lentiviral transduction of PCs with a green fluorescent protein (GFP) reporter to facilitate their visualization in 3D muscles.

1.5.2 Generation of hiPSC-derived 3D artificial skeletal muscles (steps 28-128)

To support 3D assembly of hiPSC derivatives for skeletal muscle bioengineering, we adapted a platform previously developed for cardiac tissue engineering[64] (EHT Technologies). Teflon spacers and PDMS racks (figure 2A) are sterilised by autoclaving. Additionally, 2% agarose in sterile PBS should be autoclaved and stored at 4°C. On the day (steps 29; 53; 80; 70; 97; 117), the artificial muscle biomaterial matrix (thrombin, fibrinogen[1] and growth factor-reduced (GFR) Matrigel™) should be placed on ice before proceeding with cell preparation. The 3D muscle construct measures approximately 8-10 mm in length and 2 mm in width. 3D constructs generated using exclusively hiPSC myogenic derivatives are referred to as “single lineage artificial muscles”. Constructs containing myogenic progenitor cells and one or more cell lineages, such as neural and/or endothelial and/or pericyte cells are referred to as bi-, tri-, tetra- depending on the number of lineages included or more generally as multilineage artificial skeletal muscles. Here we detail the generation of bi-lineage artificial skeletal muscles from hiPSC-derived myogenic cells and motoneuron progenitors. For tri-lineage muscles, we provide an example using inducible myogenic cells, ECs and PCs. For tetra-lineage muscle we use myogenic cells, ECs, PCs and motoneuron progenitors. Our protocol has a modular design, allowing multiple combinations depending on experimental design and desired outcomes. Different medium combinations are detailed in the materials and reagents section. Single lineage muscles last on average up to 10 days in culture whilst multilineage constructs have a longer stability in culture; up to 24 days tested, but likely extendable if required. We have successfully generated hiPSC-derived artificial skeletal muscles of different cellular complexity from multiple (>10) healthy donor and dystrophic hiPSCs (e.g., from Duchenne, *LMNA*-related and limb girdle 2D muscular dystrophies).

1.5.3 Characterisation of hiPSC-derived 3D artificial skeletal muscles (steps 129-202)

Artificial skeletal muscles need to be characterised before proceeding to downstream applications. We categorise these experiments into 3 different tiers (figure 3). Tier 1 characterisation include essential

experiments to be carried out with every artificial skeletal muscle to confirm the successful generation of single or multilineage constructs. Immunolabelling using lineage-specific markers is required to confirm the specific hiPSC derivatives included in the artificial skeletal muscles (figure 3A). We recommend using: MyHC or Titin to identify myofibres; SMI-32 for motor neurons; CD31 for ECs. To facilitate detection of PCs, we suggest staining for SM22 (an actin-binding protein of the calponin family) post purification to confirm their identity, followed by labelling with a fluorescent reporter prior to incorporation in the hydrogel (e.g., with a PGK-GFP lentivector). Tier 2 (desirable) experiments include gene and protein expression analyses, as well as functional assays such as calcium dynamics (figure 3B). Tier 3 (advanced) experiments are usually restricted to specific applications of the 3D muscle constructs and include advanced imaging (such as electron or lightsheet microscopy), inclusion and assessment of a stem/progenitor pool, contractility and implantation/biocompatibility experiments (figure 3C; supplementary videos 3 and 4).

For mRNA expression analysis and western blotting, conventional methods of RNA and protein extraction from tissues are used (figure 3B). To preserve morphology for immunofluorescence we recommend fixing muscle constructs in 4% paraformaldehyde (PFA) for 8 hours (or, alternatively, 1% PFA overnight) before removing from the PDMS posts. Staining samples immediately following PFA fixation is recommended, although fixed artificial muscles can be kept at 4°C in PBS for up to 1 month with good outcomes. We recommend reducing 4% PFA fixation to 3 hours for nuclear immunolabelling and 3D reconstruction. Incubation times of antibodies in permeabilization buffer are longer than normally used for monolayer cultures to enable better antibody penetration and washes. Artificial muscles are then mounted on slides with a concave depression and imaged using confocal microscopy (figure 3A). 3D muscle can also be paraffin-embedded and sectioned for Immunohistochemistry[1] although this is beyond the scope of this protocol.

Lightsheet microscopy can be conducted to study 3D morphology of the artificial skeletal muscles, their cell distribution and localisation of proteins in different areas of the construct (figure 3C; supplementary video 4). To test for functional myofibres within the artificial muscles we administered caffeine to induce calcium transients via sarcoplasmic reticulum calcium release and visualised them using Fluo-4 AM, which binds to released calcium, exhibiting fluorescence which was recorded using a confocal microscope (figure 3C).

Finally, assessment of muscle contraction *in vitro* and implantation of the constructs *in vivo* in mice enable study of more complex phenomena and readouts, such as maturation and function of sarcomeres, force production, fragility of myofibers and vascularization or innervation of the 3D muscles from host (in this protocol we limit assessment of contractility to *in vitro* assays, as functional *in vivo* testing requires more specialised expertise and equipment/infrastructure).

1.5.4 Applications

1.5.4.1 Disease modelling (steps 224-234)

We validated the use of our 3D artificial skeletal muscle system to recapitulate key cellular hallmarks of laminopathies such as abnormal nuclear morphology. We have also shown that 3D constructs have a superior resolution to model those nuclear phenotypic readouts compared to monolayer cultures and discovered that nuclear elongation is a prominent and measurable phenotypic outcome of myonuclei for possible *in vitro* therapy testing[2]. 3D hiPSC-derived artificial skeletal muscles and 2D myotubes from laminopathy patients were immunolabelled for Lamin A/C, and a sarcomeric protein (e.g., Titin). Samples were then imaged using confocal microscopy and the myonuclei three-dimensionally reconstructed and measured. For optimal imaging, we recommend acquiring z-planes every 0.5 µm. Image stacks are then imported into Imaris® to generate 3D reconstructions of myonuclei (figure 4A-4E; supplementary videos 5 and 6). Reconstruction is performed by setting the threshold to detect objects in the field and selecting the surfaces to exclude undesired items from rendering (please refer to supplementary protocol 1 for a stepwise guide on 3D nuclear reconstruction). Here, we describe measurement of the major axis length, as an example of an objective outcome measure (figure 4F and supplementary figure 1). These 3D reconstructions can also be used to explore other features of nuclear morphology (e.g., sphericity, area, volume) and positioning (e.g., distance between nuclei).

1.5.4.2 Additional Applications: testing gene therapy vectors and *in vivo* studies

Key applications of 3D cultures and organoid-like platforms beyond disease modelling include drug/therapy testing, toxicity studies and *in vivo* implantation for tissue replacement. Here we provide examples of further applications of this platform for *in vitro* therapy testing and *in vivo* studies. Specifically, we show that hiPSC-derived 3D muscles can be used to monitor expression in live human tissues *in vitro* and in real-time of: 1) a non-integrating, non-viral vector used in pre-clinical gene and cell therapy studies for DMD (DYS-HAC: a human artificial chromosome containing and expressing the full dystrophin gene locus[65]) (figure 5A); 2) adeno associated viral vectors (AAV) serotypes (AAV9) used in neuromuscular gene therapy clinical trials[66] (Figure 5B). Notably, the latter example highlighted dose-response correlation to different transgene dose and set the foundation for further work investigating cell and lineage specificity of different AAVs serotypes for precision medicine. Moreover, we also provide proof-of-principle for use of this 3D platform to evaluate advanced therapies beyond AAVs and show that it can be used to assess engraftment and migration of myogenic cells within isogenic 3D muscles (both derived from the same hiPSC source), providing a unique strategy to assess myogenic cell transplantation in a *quasi vivo* environment (figure 5C). Finally, we show that artificial muscles can be implanted in immunodeficient mice, where they are vascularised and integrated with the host tissue, providing the foundations for both tissue replacement strategies using this approach (figure 5D-H), as well as for *in vivo* testing of therapeutics selected from *in vitro* work.

1.6 Expertise needed to implement the protocol

Human cell culture experience, preferably with human pluripotent stem cells and with myogenic cells, as well as experience with basic molecular biology techniques, immunolabeling and confocal imaging are necessary to implement this protocol. No advanced bioengineering expertise is required given that the moulds and scaffolds are commercially available and easy to use. Alternatively, users with 3D printing expertise can produce the spacers and PDMS scaffolds in-house as recently described[67]. The protocol also relies on the use of a fluorescent cell sorter, which is usually outsourced to a specialist facility. Some possible downstream applications need specific expertise in viral vector handling and micro surgical techniques, requiring specialist training, dedicated risk assessments and standard operating procedures according to institutional guidelines.

Reagents

See 'ReagentsEquipment.pdf' in the Supplementary Files for the list of Reagents.

Equipment

See 'ReagentsEquipment.pdf' in the Supplementary Files for the Equipment list.

Procedure

1.1 Expansion of hiPSC-derived myogenic, neuronal and blood vascular cells

1.1.1 skeletal muscle cells

1.1.1.1 Transgene-based method

Transgene-based myogenic differentiation of hiPSC is based on our published protocol [56] outlined in steps 1-45. Characterisation of skeletal muscle cells is also detailed in steps 46 to 93 of the same protocol.

1. Thaw HIDEMs at 37°C until frozen clomps are no longer visible (up to 1 minute). Transfer cells to a 15 ml falcon tube containing HIDEM proliferation medium and spin at 300 rcf for 5 minutes.

2. Discard supernatant and resuspend cell pellet in HIDEM proliferation medium, plate 4-5x10^3/cm^2 cells onto an uncoated surface and incubate cells at 37°C with 5% CO_2 and 3-5% O_2.

3. After 24 hours, replace the HIDEM proliferation medium. Continue culturing cells until approximately 80% confluent prior to using for artificial skeletal muscle generation.

PAUSE POINT: HIDEMs can be expanded onto uncoated surfaces using HIDEM proliferation medium and cryopreserved using HIDEM freezing medium [56].

1.1.1.2 Transgene-free method

Small molecule-mediated hiPSC differentiation into myogenic cells was performed using a published and commercially available method[60], in which is detailed further insights into basic characterisation and testing of the differentiation capacity of the hiPSC myogenic derivatives prior to differentiation in 3D.

4. Thaw hiPSC-derived myogenic progenitors at 37°C for up to 2 minutes. Transfer cells to a 15ml falcon tube containing DMEM resuspension medium and spin at 300 rcf for 5 minutes.

5. Discard supernatant and resuspend cell pellet in Myoblast medium (Myocea), plate 4-5x10^3 cells per cm^2 onto an uncoated surface and incubate cells at 37°C with 5% CO_2 and 3-5% O_2.

6. After 24 hours, replace the Myoblast medium (Myocea). Continue culturing until 70% confluent prior to use for artificial skeletal muscle generation.

CRITICAL STEP: hiPSC-derived myogenic progenitors take 2-3 days to recover from freezing. If the culture is too confluent or cells appear flattened and large (i.e., senescing), proceed with splitting the cells either in the same flask/dish (in situ splitting) or into additional flasks/dishes to facilitate recovery and expansion.

PAUSE POINT: hiPSC-derived myogenic progenitors can be expanded onto standard uncoated tissue culture plastic grade surfaces using MYOCEA myoblast medium and cryopreserved using HIDEM freezing medium [56,60].

1.1.2 motor neurons

Derivation of motor neurons was performed using published protocols[39,40], steps from hiPSC to single cell NPCs or neurospheres are not detailed here. Characterisation of cell identity and differentiation potential of the neural progenitors according to published protocols[39,40] is required prior to use in 3D muscle cultures. The following two sections provide a basic guide to thaw and prepare cells for use in 3D muscle cultures.

1.1.2.1 Neurosphere-based method

For complete derivation of neurospheres from hiPSCs, refer to steps 3-22 and 34-39 of Stacpoole *et al.*[39]

7. Thaw neurospheres at 37°C for up to 2 minutes. Transfer to a 15 ml conical tube containing pre-warmed CDM and spin at 250 rcf for 2 minutes.

8. Discard supernatant and resuspend cell pellet in 10-12 ml of fresh neurosphere proliferation medium, seed cells in a 10 cm dish and incubate at 37°C with 5% CO_2 and 3-5% O_2.

9. After 24 hours, replace neurosphere proliferation medium. Continue culturing neurospheres by changing proliferation medium every other day.

PAUSE POINT: This cell population can then be used in bi- or tetra-lineage muscles as detailed in 1.15.2.1 and1.15.2.2

1.1.2.2 NPCs (monolayer)

10. Coat a 3.5 cm dish with 1% (vol/vol) GFR Matrigel™ or Geltrex™ at 37°C for 30 or 60 minutes respectively.

11. Thaw NPCs at 37°C for up to 2 minutes. Transfer cells to a 15ml falcon tube containing NPC proliferation medium with ROCKi (1:300) and spin at 300 rcf for 5 minutes.

12. Discard supernatant and gently resuspend cell pellet in fresh NPC proliferation medium with 1:300 ROCKi and transfer cells to the coated wells. Seed at least 2 x 10^5 NPCs per cm^2 and incubate at 37°C with 5% CO_2 and 3-5% O_2.

13. After 24 hours, change NPC proliferation medium. Continue culturing NPCs by changing NPC proliferation medium every other day.

PAUSE POINT: This cell population can then be used in bi-lineage or tetra-lineage muscles as detailed in 1.15.2.2 and 1.15.4.2.

1.1.3 Vascular endothelial cells (ECs) and pericytes (PCs)

ECs and PCs are derived from hiPSCs using a published protocol[63]. Key deviations from Orlova *et al.*[63] include cell purification by FACS and labelling of PCs with a lentivirally delivered GFP transgene to facilitate detection. Cell sorting and isolation are performed as follows:

14. Starting from step 15 of the Orlova *et al.* protocol[63], detach cells using Trypsin-EDTA.

15. Neutralise by resuspending in 20-30 ml of FACS buffer.

16. Hydrate the cell strainer with 2-3 ml of FACS buffer and immediately pass the cells through it.

17. Count cells and keep aside 2 × 10^5 cells resuspended in FACS buffer on ice for the unstained fraction to be used in calibrating the cell sorter.

18. Spin down the rest of the cells at 300 rcf for 5 minutes. Discard supernatant and incubate cell pellet with CD31 (Mouse Anti-Human CD31 antibody, TP1/15 – FITC) 1:150 for 1 hour at 4°C.

19. Resuspend cells in 10 ml of FACS sorting buffer, filter through a cell strainer and collect the cell suspension in a 15 ml tube.

20. Spin cells at 300 rcf for 5 minutes, discard supernatant and collect cell pellet for FACS.

21. Prepare 1 tube with 2 ml of EGM2 medium to collect the $CD31^-$ fraction (PCs) and 1 tube with 2 ml of EC-SFM proliferation medium to collect the $CD31^+$ fraction (ECs).

22. Prepare 0.1% gelatine-coated flasks to plate the ECs and PCs and, once the coating is complete, seed the sorted cells into the flasks in their respective medium.

PAUSE POINT: This cell population can be used for bi-lineage, tri-lineage or tetra-lineage constructs; only the latter is reported in this protocol and detailed in 1.15.4.1 and 1.15.4.2.

The following points detail an optional strategy to label PCs to facilitate their detection in 3D cultures. A lentiviral GFP transgene is suggested here, but alternative labelling strategies are likely to be equally efficacious.

23. To lentivirally transduce the PCs with a PGK-GFP cassette, follow the next steps: plate 1×10^5 PCs in a 35-mm dish in EGM2 medium.

24. When cells are fully attached to the culture dish, thaw the PGK-GFP lentiviral vector on ice and calculate the volume necessary to transduce cells with a multiplicity of infection (MOI) of 1, 5 and 10. The following formula can be used for this purpose: µl of virus needed = number of cells to infect × desired MOI/titre of virus (in ml) × 1000.

25. Dilute the PGK-GFP lentiviral vector in 1 ml of EGM2 medium supplemented with 1 µl of polybrene.

26. Remove the medium from the 35-mm dish of PGK-GFP PCs and replace it with the 1 ml of viral suspension. Incubate cells for 12 hours at 37°C with 5% CO_2 and 3-5% O_2.

27. Remove medium and wash cells twice with PBS. Subsequently, add fresh EGM2 medium and incubate cells at 37 °C with 5% CO_2 and 3-5% O_2.

PAUSE POINT: This cell population can be used for bi-lineage, tri-lineage or tetra-lineage constructs; only the latter is reported in this protocol and detailed in 1.15.4.1 and 1.15.4.2

1.2 Making hiPSC-derived artificial skeletal muscles

1.2.1 Single lineage artificial muscle

1.2.1.1 Single lineage artificial muscle by transgene-based method

CRITICAL STEP: at least 24 hours prior to beginning the protocol, ensure that the Teflon spacers and PDMS post racks are cleaned and autoclaved and that an adequate amount of 2% agarose, thrombin, fibrinogen, Matrigel™ and aprotinin aliquots are available (see Calculation Sheet, Reagent Setup and Equipment Setup)

28. Expand HIDEMs (from step 3) to 80% confluence prior to making hydrogels.

PAUSE POINT: 10^6 cells per hydrogel are required, we suggest expanding HIDEMs in T175 flasks until the area is fully covered.

29. On the day, add fresh medium supplemented with ROCKi (1:300) to the HIDEMs and incubate for 2 hours at 37°C with 5% CO_2 and 18% O_2.

30. Place thrombin and Matrigel™ aliquots on ice to thaw. Fibrinogen aliquots can be kept at RT. Label one 1.5ml Eppendorf tube per condition/cell line and place on ice.

31. Prepare an appropriate amount of HIDEM proliferation medium prepared with heat inactivated FBS to resuspend cells to be embedded in hydrogels. Volume needed depends on the number of hydrogels. For a total of 20 constructs, we suggest preparing 5 ml.

32. After two hours of incubation with HIDEM proliferation medium and 1:300 ROCKi, discard medium, wash once with PBS and cover cells with trypsin-EDTA. Incubate for up to 5 minutes to detach cells.

33. Neutralise trypsin-EDTA using HIDEM proliferation medium and count cells.

34. Centrifuge the required number of cells at 300 rcf for 5 minutes. Discard supernatant and keep cell pellet on ice.

35. Add 1.5 ml of agarose to each well of a 24-well plate that will house a 3D artificial muscle, place the spacers into the wells and allow to cool.

CRITICAL STEP: ensure spacers are placed correctly to avoid spillage of cells and biomaterial mixture at a later stage.

36. Add minimal volume of HIDEM proliferation medium prepared with heat inactivated FBS to resuspend the cell pellet. We suggest adding 50-100 µl of medium depending on the number of hydrogels (see Calculation Sheet in Supplementary Information for details).

37. Aspirate the total volume using a P200, transfer content to a 1.5 or 2ml tube/vial and note the cell suspension volume.

CRITICAL STEP: be accurate when assessing the volume, ensure that there are no bubbles in the tip when aspirating the cell suspension. If a high number of hydrogels are generated, this step can be repeated to

collect as many cells as possible from the tube but make a note of the volume of suspension transferred to the Eppendorf tube.

38. Add Matrigel™ to the cell suspension, homogenise the mixture and note the added volume (see Calculation Sheet in Supplementary Information for details).

39. For each condition/cell line, calculate the final master mix volume that will be in the Eppendorf tubes. Sum the volumes of cells calculated in step 36, Matrigel™ in step 38, and the volumes of fibrinogen and thrombin to be later added (see Calculation Sheet in Supplementary Information for details).

CRITICAL STEP: The final master mix should consist of the number of 3D muscle constructs needed plus one extra construct. Assume the volume of one construct will be lost.

40. Subtract the value calculated in step 39 from the total volume of the master mix and HIDEM proliferation medium prepared with heat inactivated FBS corresponding to the value obtained.

41. Take the required volume of fibrinogen using a P200 pipette (see Calculation Sheet in Supplementary Information for details), quickly dispense it and immediately after, resuspend the cell mixture with a P1000 pipette at least 10 times so that the fibrinogen to avoid forming clumps.

CRITICAL STEP: thaw fibrinogen at 37 °C for at least 3 minutes. This will ensure that the fibrinogen is less viscous for more accurate pipetting.

CRITICAL STEP: prepare both P200 and P1000 pipettes with tips attached prior to step 41, to be as quick as possible to prevent fibrinogen clumps forming, leading to defective constructs.

CRITICAL STEP: when releasing the fibrinogen into the Eppendorf tube, do not touch the cell suspension with the pipette tip otherwise clump could form blocking the tip.

? TROUBLESHOOTING

42. Remove the spacers from the agarose and place the PDMS post rack into the moulds.

CRITICAL STEP: make sure both PDMS posts are positioned correctly into the moulds before proceeding with the next steps.

43. Homogenise the content of the Eppendorf tube. Take 113 µl of the mixture and add it to a single thrombin aliquot (see Calculation Sheet in Supplementary Information for details). Quickly mix by pipetting up and down 1-2 times and decant content between the PDMS posts filling the gap.

CRITICAL STEP: make sure that the mixture is quickly mixed with thrombin for a maximum of two times to prevent formation of fibrin in the tube or tip. Make sure no bubbles are formed.

CRITICAL STEP: do not push the pipette until the end as bubbles might be released into the mixture which might result in potential premature snapping of the muscle constructs.

44. Cover the plate and place inside the incubator at 37 °C with 5% CO_2 and 18% O_2 for 2 hours.

45. Prepare a solution with normal HIDEM proliferation medium supplemented with aprotinin (1:1000) to prevent fibrin degradation. Add 1.5 ml per well of a new 24-well plate.

46. Add 1 ml DMEM per well on top of the hydrogels and incubate at 37 °C with 5% CO_2 and 18% O_2 for 10 minutes. This facilitates dislodging the hydrogels from the 2% agarose moulds.

47. Transfer the PDMS post racks with hydrogels to the 24-well plate containing the prewarmed medium prepared in step 45. Incubate constructs at 37 °C with 5% CO_2 and 18% O_2 for 48 hours.

48. Change medium with HIDEM proliferation medium supplemented with 1 µM 4-OH tamoxifen and aprotinin (1:1000) (first tamoxifen pulse).

49. After 24 hours, change medium with HIDEM differentiation medium supplemented with 1 µM 4-OH tamoxifen and aprotinin (1:1000) (second tamoxifen pulse).

50. Keep the hydrogels in culture until day 9, change medium every other day with HIDEM differentiation medium supplemented with aprotinin (1:1000).

? TROUBLESHOOTING

51. Upon completion of differentiation, proceed to functional assays: steps 185-202 for electrical stimulation contraction or steps 203-223 for calcium transients. Alternatively, fix the gels in 4% PFA at 4°C for 3 hours to reconstruct the nuclei in 3D or 8 hours to perform regular confocal imaging and continue with steps 129-135 for immunofluorescence. Or proceed directly to steps 161-171 for mRNA expression analysis and steps 172-184 for western blotting. Constructs to be used for mRNA expression analysis and western blotting can be snap frozen in a conical microtube in liquid nitrogen and stored in -80 °C.

1.2.1.2 Single lineage artificial muscle by transgene-free method

CRITICAL STEP: at least 24 hours prior to beginning the protocol, make sure that the Teflon spacers and PDMS post racks are cleaned and autoclaved and that an adequate amount of 2% agarose, thrombin, fibrinogen, Matrigel™ and aprotinin aliquots are available (see Calculation Sheet, Reagent Setup and Equipment Setup)

52. Expand the hiPSC-derived myogenic progenitors (from step 6) to 75% confluence prior to making hydrogels.

PAUSE POINT: 10^6 cells per hydrogel are required, we suggest expanding the myogenic progenitors in T175 flasks until they reach ~80% confluency.

53. On the day, pre-treat the hiPSC-derived myogenic progenitors with 1:300 ROCKi for 2 hours at 37 °C with 5% CO_2 and 18% O_2.

54. Place thrombin and Matrigel™ aliquots on ice to thaw. Fibrinogen aliquots can be kept at RT. Label one 1.5ml Eppendorf tube per condition/cell line and place them on ice.

55. Prepare an appropriate amount of heat inactivated MYOCEA myoblast medium to resuspend cells to be embedded in hydrogels. Volume needed depends on the number of hydrogels to be made. For example, 5 ml of inactivated MYOCEA myoblast medium is sufficient to make 20 constructs.

CRITICAL STEP: as MYOCEA myoblast medium contains horse serum, it needs to be inactivated to prevent interference with the polymerising factors, which would lead to an impaired hydrogel structure. Inactivation can be achieved by incubating a MYOCEA myoblast medium-containing 1.5 ml Eppendorf tube at 56 °C for 30 minutes.

56. Detach cells by adding 2 ml of trypsin-EDTA in each T175 flasks and incubating at 37 °C with 5% CO_2 and 3-5% O_2 for up to 5 minutes.

57. Neutralise the trypsin-EDTA with DMEM resuspension medium and count cells.

58. Follow steps 34-44 to generate the transgene-free artificial muscles.

59. Prepare a solution with normal MYOCEA myoblast medium and aprotinin (1:1000). Add 1.5ml per well of a new 24-well plate.

60. Transfer the PDMS post racks with hydrogels to the 24-well plate containing prewarmed MYOCEA myoblast medium and incubate 37 °C with 5% CO_2 and 18% O_2 for 48 hours.

61. After 2 days, switch to MYOCEA fusion medium supplemented with aprotinin (1:1000) to begin terminal differentiation.

62. Keep hydrogels in culture for at least 7 days, change medium every other day with MYOCEA fusion medium supplemented with aprotinin (1:1000).

63. To generate constructs containing PAX7+ cells, follow steps 53-60 and use early passage hiPSC-derived myogenic progenitors (cells at day 2 of the MYOCEA myoblast stage[60]).

64. Transfer hydrogels to prewarmed MYOCEA myoblast medium supplemented with aprotinin (1:1000).

65. Change MYOCEA myoblast medium with aprotinin (1:1000) every other day for the subsequent 5 days.

66. On day 6, switch to MYOCEA fusion medium and aprotinin (1:1000) to begin terminal differentiation.

67. Keep the hydrogels in culture until day 14 adding fresh MYOCEA fusion medium supplemented with aprotinin (1:1000) every other day.

? TROUBLESHOOTING

68. Upon completion of differentiation, proceed to functional assays: steps 185-202 for electrical stimulation contraction or steps 203-223 for calcium transients. Alternatively, fix the gels in 4% PFA at 4°C for 3 hours to reconstruct the nuclei in 3D or 8 hours to perform regular confocal imaging and continue with steps 129-135 for immunofluorescence. Or proceed directly to steps 161-171 for mRNA expression analysis and steps 172-184 for western blotting. Constructs to be used for mRNA expression analysis and western blotting can be snap frozen in a conical microtube in liquid nitrogen and stored in -80 °C.

1.2.2 hiPSC-derived bi-lineage muscles containing skeletal myofibres and motor neurons

1.2.2.1 Bi-lineage artificial muscle using neurospheres

69. Generate the desired number of single lineage artificial muscles as described in steps 28-47.

70. Prepare a mixture of 113 µl of MegaCell DMEM and 14 µl of thrombin. Keep on ice.

71. Hold the PDMS post in hand and deposit 5 µl of fibrinogen on the artificial muscle.

CRTICAL STEP: warm up fibrinogen by hand or in a bead bath for 3 minutes to reduce its viscosity to pipette more accurately.

72. Collect at least 3 of the neurospheres described in step 8 using a 20 µl pipette and add within the fibrinogen drop.

CRITICAL STEP: when collecting the neurospheres, aspirate as little neurosphere proliferation medium as possible to avoid negative interactions with fibrinogen.

73. Immediately add 7 µl of the MegaCell DMEM and thrombin solution (from step 70) on top of the neurospheres and fibrinogen mixture. Wait 3 minutes to allow formation of fibrin.

74. Transfer hydrogels to a 24-well plate containing 1.5 ml of neurosphere bi-lineage differentiation medium supplemented with 1 µM of 4-OH tamoxifen, 5 µg/ml heparin and aprotinin (1:1000) per well and place in the incubator at 37 °C with 5% CO_2 and 18% O_2.

75. After 24 hours, perform a full medium change with fresh neurosphere bi-lineage differentiation medium supplemented with 1 µM of 4-OH tamoxifen and aprotinin (1:1000).

76. Change neurosphere bi-lineage differentiation medium supplemented with aprotinin (1:1000) every other day until day 6.

77. From day 6, change medium with neurosphere bi-lineage differentiation medium supplemented with 1 μM purmorphamine (PM) and aprotinin (1:1000) every other day until day 14.

? TROUBLESHOOTING

78. Upon completion of differentiation, proceed to functional assays: steps 185-202 for electrical stimulation contraction or steps 203-223 for calcium transients. Alternatively, fix the gels in 4% PFA at 4°C for 3 hours to reconstruct the nuclei in 3D or 8 hours to perform regular confocal imaging and continue with steps 129-135 for immunofluorescence. Or proceed directly to steps 161-171 for mRNA expression analysis and steps 172-184 for western blotting. Constructs to be used for mRNA expression analysis and western blotting can be snap frozen in a conical microtube in liquid nitrogen and stored in -80 °C.

1.2.2.2 Bi-lineage artificial muscle using NPCs (as single cells, not in neurospheres)

79. Thaw and culture the HIDEMs and hiPSC-derived NPCs as described in steps 1-3 and 10-13, respectively, until they reach 90-100% confluence.

80. To prepare reagents for making the bi-lineage complex artificial muscles, follow the steps 30-31.

81. Pre-treat both NPCs and HIDEMs with ROCKi (1:300) for 2 hours prior to making the bi-lineage complex muscle constructs.

82. Collect the required cell numbers of HIDEMs by following step 32.

PAUSE POINT: A mix of 70% HIDEMs and 30% NPCs (e.g., 7×10^5 and 3×10^5 per hydrogel respectively) is required.

83. Incubate NPCs with 0.5 mM EDTA at 37°C for 5 minutes and then remove the EDTA solution. Add N2B27 medium.

84. Detach cells from the bottom of the dish by gently pipetting up and down the N2B27 medium. When cells have detached, collect in a 15ml falcon tube and count NPCs.

85. Upon mixing the appropriate number of the two cell types, spin cell mixture at 300 rcf for 5 minutes.

86. Discard supernatant and add a minimal volume of NPC bi-lineage proliferation medium containing heat inactivated FBS to resuspend the cell mixture. We suggest adding 50-100 μl of heat inactivated NPC bi-lineage proliferation medium depending on the number of hydrogels (see Calculation Sheet in Supplementary Information for details).

87. Note cell mixture volume, then aspirate the contents using a P200 pipette and transfer to the respective Eppendorf tubes.

88. Follow steps 38-44 to generate the bi-lineage constructs using NPC bi-lineage proliferation medium with heat inactivated FBS.

89. Transfer hydrogels into prewarmed NPC bi-lineage proliferation medium supplemented with aprotinin (1:1000) and incubate the hydrogels at 37 °C with 5% CO_2 and 18% O_2 for 48 hours.

90. Change medium with NPC bi-lineage proliferation medium supplemented with 1 µM 4-OH tamoxifen, 0.1 µM y-secretase inhibitor and aprotinin (1:1000) (first tamoxifen pulse).

91. After 24 hours, change medium with NPC bi-lineage differentiation medium with 1 µM 4-OH tamoxifen, 0.1 µM y-secretase inhibitor and aprotinin (1:1000) (second tamoxifen pulse).

92. Hydrogels are kept in culture performing daily changes of NPC bi-lineage differentiation medium containing 0.1 µM y-secretase inhibitor and aprotinin (1:1000).

93. On day 5, perform an NPC bi-lineage differentiation medium change with supplementation of 0.1 nM agrin and aprotinin (1:1000). Perform the same NPC bi-lineage differentiation medium change using 0.5 nM and 1 nM of agrin on day 6 and 7, respectively.

94. From day 8, change medium with NPC bi-lineage differentiation medium supplemented with aprotinin (1:1000) and 1 nM of agrin daily until day 14 (or beyond this date for longer cultures).

? TROUBLESHOOTING

95. Upon completion of differentiation, proceed to functional assays: steps 185-202 for electrical stimulation contraction or steps 203-223 for calcium transients. Alternatively, fix the gels in 4% PFA at 4°C for 3 hours to reconstruct the nuclei in 3D or 8 hours to perform regular confocal imaging and continue with steps 129-135 for immunofluorescence. Or proceed directly to steps 161-171 for mRNA expression analysis and steps 172-184 for western blotting. Constructs to be used for mRNA expression analysis and western blotting can be snap frozen in a conical microtube in liquid nitrogen and stored in -80 °C.

1.2.3 hiPSC-derived tri-lineage artificial muscles containing myofibres and blood vascular endothelial cells and pericytes

96. Culture HIDEMs (steps 1-3), ECs (step 21) and PCs (step 27) in T175 flasks containing HIDEMs proliferation, EC-SFM and EGM2 medium, respectively, until they reach desired confluence (see section 1.14).

97. To prepare reagents for making the tri-lineage complex artificial muscles, follow steps 30-31.

98. Pre-treat ECs, PCs and HIDEMs with ROCKi (1:300) 2 hours prior to making the tri-lineage complex muscle constructs.

99. Obtain the required cell numbers of HIDEMs, ECs and PCs by following steps 32-34. Make sure to use the appropriate medium for each cell type for inactivation of trypsin.

PAUSE POINT: A mix of 70% HIDEMs (7×10^5) and 30% vascular cells (6% ECs and 24% PCs (6×10^4 ECs and 2.4×10^5 PCs)) per hydrogel is required.

100. Spin cells at 300 rcf for 5 minutes, discard supernatant and resuspend in appropriate volume of tri-lineage proliferation medium with heat inactivated FBS.

101. Follow steps 38-44 to generate the tri-lineage constructs using tri-lineage proliferation medium with heat inactivated FBS.

102. Transfer the PDMS post rack with hydrogels into prewarmed tri-lineage proliferation medium containing aprotinin (1:1000) and incubate the hydrogels at 37 °C with 5% CO_2 and 18% O_2 for 48 hours.

103. Change medium with tri-lineage proliferation medium supplemented with 1 µM of 4-OH tamoxifen and aprotinin (1:1000) (first tamoxifen pulse).

104. After 24 hours, change the medium with tri-lineage differentiation medium supplemented with 1 µM of 4-OH tamoxifen and aprotinin (1:1000) (second tamoxifen pulse).

105. Keep the hydrogels in culture until day 17 performing a full medium change with tri-lineage differentiation medium supplemented with aprotinin (1:1000) every other day.

? TROUBLESHOOTING

106. Upon completion of differentiation, proceed to functional assays: steps 185-202 for electrical stimulation contraction or steps 203-223 for calcium transients. Alternatively, fix the gels in 4% PFA at 4°C for 3 hours to reconstruct the nuclei in 3D or 8 hours to perform regular confocal imaging and continue with steps 129-135 for immunofluorescence. Or proceed directly to steps 161-171 for mRNA expression analysis and steps 172184 for western blotting. Constructs to be used for mRNA expression analysis and western blotting can be snap frozen in a conical microtube in liquid nitrogen and stored in -80 °C.

1.2.4 Tetra-lineage artificial muscles containing myofibres, motor neurons and vascular cells

1.2.4.1 Option A - Neurospheres:

107. Generate a tri-lineage construct following steps 96-101.

108. After 1 hour of polymerisation, decant 3-6 neurospheres on top of each hydrogel using 10 µl of fibrinogen.

109. Transfer the PDMS post racks with hydrogels into prewarmed neurosphere tetra-lineage proliferation medium containing aprotinin (1:1000) and keep at 37 °C with 5% CO_2 and 18% O_2 for 48

hours.

110. Change medium with neurosphere tetra-lineage proliferation medium supplemented with 1 μM of 4-OH tamoxifen and aprotinin (1:1000) (first tamoxifen pulse).

111. After 24 hours, change medium with neurosphere tetra-lineage differentiation medium supplemented with 1 μM of 4-OH tamoxifen and aprotinin (1:1000) (second tamoxifen pulse).

112. Perform medium changes every 2 days with neurosphere tetra-lineage differentiation medium and aprotinin (1:1000) until day 7.

113. From day 8, perform full medium changes with fresh neurosphere tetra-lineage differentiation medium supplemented with 1 μM of PM and aprotinin (1:1000) every other day.

? TROUBLESHOOTING

114. Upon completion of differentiation, proceed to functional assays: steps 185-202 for electrical stimulation contraction or steps 203-223 for calcium transients. Alternatively, fix the gels in 4% PFA at 4°C for 3 hours to reconstruct the nuclei in 3D or 8 hours to perform regular confocal imaging and continue with steps 129-135 for immunofluorescence. Or proceed directly to steps 161-171 for mRNA expression analysis and steps 172-184 for western blotting. Constructs to be used for mRNA expression analysis and western blotting can be snap frozen in a conical microtube in liquid nitrogen and stored in -80 °C.

1.2.4.2 Option B - Neural Progenitor Cells (NPCs):

115. Culture HIDEMs (steps 1-3), ECs (step 21) and PCs (step 27) in T175 flasks containing HIDEMs proliferation, EC-SFM and EGM2 medium respectively until they reach appropriate confluence.

116. Culture NPCs as described in steps 10-13 until the full confluence of at least one 6-well plate is reached.

117. To prepare reagents for making the tetra-lineage artificial muscles, follow steps 28-30.

118. Pre-treat ECs, PCs, HIDEMs and NPCs with ROCKi (1:300) for 2 hours prior to making the tetra-lineage constructs.

119. Obtain the required numbers of HIDEMs, ECs and PCs by following steps 32-34. Make sure to use the appropriate medium for each cell type for inactivation of trypsin.

120. Count the number of NPCs.

121. Mix the 4 different cell types in the required ratio and spin the cell mixture at 300 rcf for 5 minutes.

PAUSE POINT: 70% myogenic cells (7×10^5), 15% vascular cells (10% ECs and 5% PCs (1×10^5 ECs and 5×10^4 PCs)) and 15% NPCs (1.5×10^5).

122. Discard supernatant and resuspend cells in a minimal volume of NPC tetra-lineage proliferation medium containing heat inactivated FBS. We suggest adding 50-100 µl of heat inactivated NPC tetra-proliferation medium depending on the number of hydrogels (see Calculation Sheet in Supplementary Information for details).

123. Follow steps 38-44 to generate hydrogels using NPC tetra-lineage proliferation medium with heat inactivated FBS.

124. Transfer the PDMS post racks with hydrogels into prewarmed NPC tetra-lineage proliferation medium containing aprotinin (1:1000) 2 hours after embedding the cells into fibrin hydrogels and incubate at 37 °C with 5% CO_2 and 18% O_2 for 48 hours.

125. Change medium with NPC tetra-proliferation medium supplemented with 1 µM of 4-OH tamoxifen and aprotinin (1:1000) (first tamoxifen pulse).

126. After 24 hours, change medium with NPC tetra-lineage differentiation medium supplemented with 1 µM of 4-OH tamoxifen and aprotinin (1:1000).

127. Keep the gels in culture until day 24 and add replace NPC tetra-differentiation medium supplemented with aprotinin (1:1000) every other day.

? TROUBLESHOOTING

128. Upon completion of differentiation, proceed to functional assays: steps 185-202 for electrical stimulation contraction or steps 203-223 for calcium transients. Alternatively, fix the gels in 4% PFA at 4°C for 3 hours to reconstruct the nuclei in 3D or 8 hours to perform regular confocal imaging and continue with steps 129-135 for immunofluorescence. Or proceed directly to steps 161-171 for mRNA expression analysis and steps 172184 for western blotting. Constructs to be used for mRNA expression analysis and western blotting can be snap frozen in a conical microtube in liquid nitrogen and stored in -80 °C.

1.3 Characterisation of 3D artificial muscle

Please note that this section is organised based on technical workflows and not according to the tiers mentioned in figure 3, which we advise the reader to follow.

1.3.1 Morphological characterisation

1.3.1.1 Immunofluorescence

129. Remove the fixed hydrogels from the PDMS posts using the tweezers and transfer to a conical tube containing the blocking buffer. The artificial muscles can also be stored in PBS at 4°C for up to 1 week.

CRITICAL STEP: carefully run the tweezers along one post and remove one end of the construct. Follow the same procedure for the other end of the construct. Do not touch the central portion of the hydrogels

with tweezers as this could damage the construct.

130. Blocking and permeabilisation: incubate fixed artificial muscles in blocking buffer for 6 hours at 4°C to block non-specific binding.

131. Remove the blocking buffer and incubate with primary antibody/antibodies in permeabilisation buffer at 4 °C overnight.

132. Remove the primary antibody and wash the samples six times for 60 minutes each in TBS 1X at RT.

133. Incubate samples with secondary antibody/antibodies and Hoechst in permeabilisation buffer at 4 °C overnight on a plate shaker.

134. Remove the secondary antibody and wash samples six times for 60 minutes each in TBS 1X at RT on a plate shaker.

CRITICAL STEP: antibodies conjugated with fluorophores are light sensitive, so step 134 on must be performed while protecting the samples from light (e.g., by covering samples with aluminium foil).

135. Once the immunofluorescence is completed, either proceed with confocal (steps 136-145) or light-sheet microscopy (steps 146-160).

PAUSE POINT: immunohistochemistry can also be performed on sectioned constructs to further characterise and image tissue architecture. For further details see Maffioletti *et al.,* 2018 [1].

1.3.1.2 Confocal and light sheet microscopy

1.3.1.2.1 Confocal microscopy

136. For confocal imaging, mount the immunolabelled hydrogels (129-135) on microscope glass slides with a concave depression using 2-3 drops of fluorescence mounting medium.

137. Gently place a coverslip on top of the sample and let dry for 20 minutes.

CRITICAL STEP: make sure the constructs are positioned in the middle of the concave depression, covered with fluorescence mounting medium while avoiding bubbles.

PAUSE POINT: to seal the coverslip to the glass slide, nail polish can be applied to the sides of the coverslip and left to dry for 20 minutes at RT.

138. Place the slide onto the stage of the confocal microscope and use the following settings: 63× objective, scanning parameters: 1,024x1,024, speed of 600, zoom factor 1.0, frame average of 3 and z-stack spacing of 1.5 µm. Set the fluorescent filters as required and their intensity, which should not exceed a wavelength of 30 nm.

139. For 3D reconstruction of nuclei, use the same settings as in the step 138 but use a zoom factor of 1.5 and z-stack spacing of 0.5 µm.

140. As the 63× is an immersion objective, dispense two drops of the immersion oil on top of the coverslip prior to imaging.

141. Lower the objective until it contacts the immersion oil. Use the ocular to further lower the objective until the sample is in focus.

142. Select the field to image and set the start and end of the Z position moving the objective up and down across the sample. This will represent the portion of artificial muscle over which the z-stack will be collected.

PAUSE POINT: we advise choosing the start/end of the Z position based on the fluorescent channel of major interest (e.g., Lamin A/C to reconstruct myonuclei)

143. For each fluorescent channel, use the LUT tool to adjust the gain and the offset to improve signal intensity and background noise respectively.

144. Initiate imaging. Upon completion of this process, use the Intensity Max Projection tool to combine stack to generate a single image to be exported as a TIFF file.

145. For 3D nuclear reconstruction, export the files in LIF format and proceed to step 224.

1.3.1.2.2 Light sheet microscopy method

The steps listed are the same as recommended by the manufacturer, with minor changes to better fit 3D artificial skeletal muscles. It is worth noting that the 3D artificial skeletal muscle is quite opaque and so the maximum depth that can be imaged is 1-2 myotubes from the exterior of the construct.

146. Prepare the light sheet fluorescence microscope by setting up the objective (5×), the filter cubes and perfusion chamber (filled with PBS to the top of the chamber windows) onto the chamber mount. Make sure the relevant filter cubes are installed.

147. Assemble the syringe into the microscope's sample holder disk with syringe adapter ring.

148. Following immunolabelling (steps 129-135), using forceps, place the construct on a 10 cm dish.

CRITICAL STEP: ensure that there is no excess of PBS but that the construct is not completely dry.

149. Dip the tip of the needle into a droplet of superglue and attach to the furthest end of the muscle construct, having a couple of millimetres of overlap between the needle and construct so that both are in tandem.

CRITICAL STEP: this step must be performed rapidly as superglue sets quickly.

150. Leave to set for 30 seconds and then attach the needle to the syringe.

151. Slowly mount the sample holder disk onto the microscope stage and into the upper opening.

152. Using the Zen software specimen locate sample tool, find and centre the specimen along the 3 axes.

153. In the acquisition tab, setup the light path by defining the necessary tracks.

154. Activate multi-view checkbox in multidimensional input field, use the now-available z-stack tool window to select the upper and lower limit of imaging using the “set first” and “set last” buttons, the interval (recommended 1 µm) and the number of rotations (recommended 3).

155. Start imaging of the constructs by clicking start experiment.

156. Once done, remove the holder disk from stage and the needle from the syringe.

157. Place sample gently onto 10 cm dish and hold needle at 30°, then quickly slide the blade on the needle to detach the superglued muscle construct from the needle.

158. Using forceps, place the construct back into PBS-filled conical tube and keep away from light.

159. The acquired image is exported in CZI file extension and imported in Arivis Vision4D software.

160. Reconstruct the 3D muscle and export images and videos.

PAUSE POINT: the same sample can be imaged by both light sheet and confocal microscopy. It is advisable to not excessively manipulate the artificial muscle to preserve its structure and to perform steps 146-160 quickly to avoid photobleaching of the fluorophores.

1.3.2 Molecular characterisation

1.3.2.1 mRNA expression analysis

161. Wash constructs 3 times with PBS, then remove hydrogels from the PDMS posts as described in step 129 and transfer to Eppendorf tubes containing 500 µl of Trizol per sample.

PAUSE POINT: if RNA extraction is not performed immediately, dry constructs can be stored in vials/tubes at -80°C for up to 6 months.

162. Homogenise for approximately 30 seconds on ice, then incubate at RT for 5 minutes.

163. Add 100 µl of chloroform per sample and shake intermittently for 3 minutes at RT.

164. Centrifuge at 12,000 rcf (or more) for 15 minutes at 4°C.

165. Transfer aqueous phase containing the RNA without touching the interphase to a new Eppendorf tube.

166. Add 250 µl of isopropanol to the aqueous phase. Incubate for 10 minutes and centrifuge at 12,000 rcf for 10 minutes at 4°C

167. Discard supernatant and add 1 ml of chilled 70% ethanol, vortex and centrifuge at 7,000 rcf for 5 minutes at 4°C.

168. Discard supernatant, air dry the RNA pellet for few minutes at RT and resuspend in 20 µl of nuclease free water and measure the RNA yield and purity using a Nanodrop.

169. Use 1 µg of RNA to retro-transcribe to cDNA with the ImProm-II™ Reverse Transcription System kit.

170. Perform a PCR for a housekeeping gene (e.g. *GADPH, TBP*) to evaluate the efficacy of the retro-transcription reaction.

171. Perform Quantitative Real Time-PCR to assess gene expression (e.g., for myogenic factors) with the GoTaq® qPCR Master Mix kit following manufacturer's directions using a BioRad CFX96 machine.

1.3.2.2 Western Blot

172. Add ice-cold lysis buffer to cell pellets (50 µl/ sample) or 3D muscle constructs (200 µl/ sample).

173. Homogenise samples for 30 seconds using the Ultra-Turrax homogeniser (power level 5, 20,500 rpm) on ice.

174. Incubate samples on ice for 30 minutes, mixing very well every 5-10 minutes and then centrifuge at 12,000 rcf for 10 minutes at 4°C.

175. Collect supernatant (containing proteins) and use to determine sample concentration. Protein suspensions can be stored at -80°C indefinitely.

176. Prepare a standard curve using bovine serum albumin (BSA) 8 mg/ml to 0.5 mg/ml and quantify samples using the colourimetric reaction DC Protein Assay and the iMark microplate reader.

177. Heat samples at 98°C for 5 minutes in reducing loading Laemmli buffer.

PAUSE POINT: if samples are stored at -80°C, thaw on ice before use.

178. Load a molecular weight ladder and 30 µg of protein per sample on the 4%-15% precast gel and run at 100 V for 2 - 3 hours.

179. Transfer the proteins onto the polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer system (iBlot gel transfer system) at 20 V for 7 minutes.

180. Stain the membrane using Ponceau S to confirm correct protein transfer.

181. Rinse the membrane 3 times using TBST buffer then block using milk-TBST blocking buffer for 60 minutes at RT.

182. Incubate PVDF membrane in milk-TBST blocking buffer containing primary antibodies of choice at respective concentration on a shaker overnight. Wash 3 times using TBST buffer for 10 minutes each at room temperature.

183. Incubate PVDF membrane in milk-TBST blocking buffer containing HRP-conjugated secondary antibody at respective concentration for 1 hour at RT on a shaker. Then wash 3 times using TBST buffer for 10 minutes each at RT.

184. Visualise and develop the signal by incubating the membrane with ECL reagents, according to manufacturer's instructions, and image using the chemiluminescence detector (ChemiDoc).

1.3.3 Functional characterisation

1.3.3.1 Contractility assay

185. Assemble the imaging set up: the plate holder, the lamp and the inverted Basler camera and connect to computer.

186. On the computer, create a new folder (with subfolders) to save the recordings, and start the Pylon program and select the Basler ace camera.

187. Select "Windows" and then "Recording setting" and set the acquisition speed to 25 fps (records a frame every 40 milliseconds), the recording time to 20 seconds and select the desired output folder.

188. Prepare the electric stimulator and connect it to the autoclaved Pacing electrodes.

189. Add 500 µl of warm differentiation medium to each well to reach a total volume of 2 ml per well.

190. Place on the plate-holder and remove lid. Position the light above the skeletal muscle construct and position the camera under it.

191. Gently mount the carbon electrodes on the well containing the muscle construct. Dip the two electrodes into the medium. Use Blu Tack® to fix the electrodes to the plate.

CRITICAL STEP: Avoid touching the constructs with the electrodes or the electrodes touching each other.

192. Switch on the electrical stimulator.

CRITICAL STEP: Make sure that the pulse is off at this stage.

193. On the pylon software, select the camera icon and record the first video of the hydrogel without an electrical stimulus; this is the baseline.

194. Set up the desired voltage, frequency on the electrical stimulator to achieve the required current.

PAUSE POINT: start with a stimulation of 2 Hz and 5 V. Increase voltage or frequency if the aim is to further challenge the muscle.

195. Select the new destination folder, turn the pulse switch to the on state and start recording the video immediately. Alternatively, start recording before turning on the stimulation to obtain the baseline and contraction from the same video.

196. Turn off the pulse switch and the stimulator.

197. Gently remove the electrodes and fix the constructs with PFA 4% for 3 hours to reconstruct the nuclei in 3D or 8 hours to perform normal confocal imaging and continue with steps 129-135 for immunofluorescence. Alternatively, proceed directly to steps 161-171 for mRNA expression analysis or steps 172-184 for western blotting. Constructs to be used for mRNA expression analysis and western blotting can be snap frozen in a conical microtube in liquid nitrogen and stored in -80 °C.

Analysis:

198. To analyse the video (e.g. supplementary video 1), install the MUSCLEMOTION plugin on ImageJ.

199. Drag the folder with the pictures to FIJI and in select “Use virtual stack” to mount the video.

200. Save the entire video.

201. Click on the MUSCLEMOTION icon. In the “analysis parameters wizard” insert the frame rate of the recording (25 fps in step: 187) and the speed to analyse the video should be set to 1 frame. Select “yes, but keep it simple” to decrease the noise in the output and detecting the reference frame and “No” for analysis of the transient time.

PAUSE POINT: Refer to the MUSCLEMOTION user manual for further information regarding the plugin and settings.

202. Select the folder to save the analysis and click on the video to analyse.

1.3.3.2 Whole construct calcium transients

203. Incubate artificial muscles anchored to the PDMS post racks in Fluo-4 Loading Buffer for 30 minutes at 37°C.

204. Wash the constructs using DMEM for 5 min.

205. Transfer constructs and posts on glass bottom dishes.

CRITICAL STEP: For high quality imaging, make sure constructs adhere to the bottom of the wells. If the construct does not touch the bottom of the plate, fill the gap with medium.

206. Set the long-term time-lapse wide-field system Nikon microscope to fluorescence excitation of 488 nm with a 10X lens. Set image acquisition every 0.333 sec for a total of 90 sec.

CRITICAL STEP: Ensure that the CO_2 and temperature settings in the chamber are correct.

207. Place the plate on the stage and gently mount the carbon electrodes on the well containing the muscle construct as described in step 188 and 191.

208. Start the time lapse acquisition. The first 10 seconds are acquired as baseline, after setting up the desired voltage and frequency, switch on the electrical stimulator.

209. At the end of recording constructs can be fixed or used for RNA/protein extraction.

Analysis:

210. Export the image series into Fiji / ImageJ

211. Click on Image and select the Stacks option. Click on Plot Z-axis profile. Save the data as xls.

1.3.3.3 Single cell calcium transients

212. With the 3D artificial muscles retained on the PDMS post racks, incubate in Fluo-4 Loading Buffer for 30 minutes at 37°C.

213. Wash the constructs using recording buffer.

214. Take the 3D artificial muscles out of medium and carefully disassemble them from the PDMS posts using fine tip forceps, place them in a 10 cm dish and submerge samples in recording buffer.

CRITICAL STEP: remove 3D artificial muscles very gently and place carefully to avoid snapping them. For the best imaging quality, make sure most of the construct body is sticking to the bottom of the plate.

215. Set the Zeiss 880 confocal LSM to fluorescence excitation of 488 nm and emission collected at >530 nm and prepare to acquire as a time series at 33 frames per second (fps).

CRITICAL STEP: make sure that the CO_2 and temperature settings in the chamber are correct.

216. Place the plate under the stage, choose the field with most aligned myotubes and perform imaging using a 10× objective.

217. Add caffeine to the recoding buffer at a final concentration of 10 mM and immediately mark the time/captured frame on the microscope software.

218. Repeat step 217 when fluorescence excitation subsides, for as many times as necessary.

219. Fix the constructs in 4% PFA at 4°C for 3 hours to reconstruct the nuclei in 3D or 8 hours to perform regular confocal imaging and continue with steps 129-135 for immunofluorescence. Alternatively, proceed to steps 161-171 for mRNA expression analysis and steps 172-184 for western blotting. Constructs to be used for mRNA expression analysis and western blotting can be snap frozen in a conical microtube in liquid nitrogen and stored in -80 °C.

Analysis:

220. Export time series from microscope and open in Fiji software (also known as ImageJ).

221. Select areas (each representing a myotube and covering a maximum of 10% of its area to avoid interfering signals from other cells) using the “Lasso” tool.

222. From the “Analyse” menu, choose “Set measurements” and select “Mean grey value”.

223. From the “Image” menu, select “Stacks”, “Plot z axis profile” and save as an xls file.

1.4 Disease Modelling: a stepwise guide to study nuclear morphology and model laminopathy-associated nuclear abnormalities using 3D artificial muscles

One of the most characteristic pathological hallmarks of laminopathies is nuclear shape abnormality. This aspect can be modelled utilising the 3D artificial muscle platform described in this protocol by reconstructing the nuclei in three-dimensions[2]. For both 3D artificial muscles and 2D classic monolayer cultures, the imaging and the three-dimensional nuclear reconstruction were performed in the same way for a more robust comparison. For more details see also Supplementary Information.

224. Import the raw (LIF) files in Imaris® 8.4.1, obtained in step 145, by clicking “Image” in the upper menu (the images will automatically appear in the “Arena” window). Images can also be arranged in folders named “Assays” or “Groups” also found in the upper menu.

225. Select the image to reconstruct, which will show under the “Surpass” window in a “3D view”. To reconstruct the nuclei, click on to “Add new surfaces” on the left-hand menu. The “Surface” window will show in the below panel. If the “Display Adjustment” window does not appear automatically, it can be opened by clicking Edit>Show Display Adjustment. This function will allow the operator to tick and untick the channels to be visualised.

? TROUBLESHOOTING

226. Algorithm: choose “Default” as a Favourite Creation Parameters and proceed by clicking on the blue arrow at the bottom of the panel.

PAUSE POINT: the operator can also generate and store Creation Parameters that can be applied to any number of grouped images.

227. Source channel: select the channel to reconstruct (e.g., the red channel indicating lamin A/C), tick the box “Smooth” and choose the “Surface Detail” value that better sets up the smoothness of the resulting area (e.g., 0.200-0.300 µm). Tick “Absolute Intensity” box as the parameter to base the thresholding on and proceed by clicking on the blue arrow at the bottom of the panel.

228. Threshold: this is based on the intensity of the immunofluorescence in the analysed image. The lower the value the more objects are detected and reconstructed within the image. By ticking the box “Enable”, it is possible to split the touching objects. Click on the blue arrow at the bottom of the panel.

CRITICAL STEP: it is advisable to set the threshold at around a value that allows the detection of low-intensity objects while preserving the natural size of the objects with higher fluorescence intensity.

229. Classify surfaces: this function allows the removal of objects that can interfere with the final results and analysis. To achieve this, several filters can be selected (e.g., number of voxels, area, volume, etc.). Use this function to remove small unspecific and partially reconstructed objects that are not relevant. Click on the green arrow on the bottom of the panel to execute the creation steps.

CRITICAL STEP: ensure most unspecific objects are removed. To achieve this goal, untick/tick the “Volume” box on the upper panel to better understand what is the specific and the unspecific signal detected.

230. Major axis length analysis: upon 3D reconstruction of the nuclei, select “Add new measurement point” in the left panel menu (where the “Add surfaces” tool is).

231. Choose “Settings” in the below panel to set the point/line shape, size and labels. Select “Pairs” for “Line mode” and tick “Distance” for “Line Labels”.

232. Click on “Edit” and choose “Surface of object” as the intersection site when adding the points. This action is performed by shift-clicking the left mouse button to add the point on to the desired object. Observe the image and use the “Navigate” function on the top right corner menu under the item “Pointer” to assess which myonucleus to measure.

CRITICIAL STEP: use the “Display adjustment” tool to assess whether a nucleus is located within or outside a myotube.

233. Choose “Select” from the top right menu to pinpoint one extremity of the myonucleus by shift-clicking the mouse left button. After selecting “Navigate” and turning the image, add the second extremity to the same nucleus. The distance between the two points will display automatically.

CRITICAL POINT: make sure the extremities are positioned in the most appropriate way by selecting “Surface”> “Colour”> “Transparency”. This function will allow a better visualisation of the line throughout the objects.

234. After measuring the desired objects in the image, select “Statistics”, click on “Detailed” tab, “Specific Values” and export the excel files including all the nuclear long axis values to the desired directory. The resulting spreadsheet will include all relevant information such as the type of object measured (e.g., surface), the outputted value, the unit of measurement as well as the object ID to enable tracking of analysed items. Examples of possible additional measures other than the major axis length, include nuclear surface area and volume.

Troubleshooting

For troubleshooting information, see Table 2 in the Supplementary files.

Time Taken

Steps 1-3. Culturing of transgene derived skeletal myogenic cells; 2-3 days

Steps 4-6. Culturing of small molecule derived skeletal myogenic cells; 2-3 days

Steps 7-9. Culturing of neurospheres; 4-6 days

Steps 10-13. Culturing of monolayer motor neurons; 2-6 days

Steps 14-27. Derivation of endothelial cells and pericytes; 4-6 days

Steps 28-68. Making single lineage artificial muscle; 9-11 days

Steps 69-78. Making bi-lineage artificial muscle with neuroshperes; 16 days

Steps 79-95. Making bi-lineage artificial muscle with NPCs; 16 days

Steps 96-106. Making tri-lineage artificial skeletal muscles; 19 days

Steps 107-128. Making Tetra-lineage artificial skeletal muscle; 26 days

Steps 129-135. Immunofluorescence characterisation of 3D artificial muscle; 2 days

Steps 136-145. Confocal imaging of 3D artificial muscle; 1-5 hours

Steps 146-160. Lightsheet imaging of 3D artificial muscle; 5-18 hours

Steps 161-171. mRNA expression analysis of 3D artificial muscle; 8-18 hours

Steps 172-184. Western blotting protein expression analysis of 3D artificial muscle; 2-3 days

Steps 185-202. Contractility assay; 15 minutes.

Steps 203-211. Whole construct calcium transients; 2-5 hours

Steps 212-223. Single cell calcium transients; 2-5 hours.

Steps 224-234. Modelling laminopathy related abnormalities; 3-15 hours (depending on the number of samples to be analysed)

Anticipated Results

In this protocol we detail the modular generation of human 3D artificial muscle constructs fully derived from pluripotent stem cells. We focused on hiPSCs as the preferential source of cells given the limited ethical constraints and non-invasiveness of the original cell population sampling, although similar results could be obtained using hESCs[1] or more conventional cell sources such as human primary or immortalised skeletal myoblasts[1,20]. We provide a stepwise guide to generate an array of 3D bioengineered muscles with incremental multicellular and multi-lineage complexity: from bundles of myofibres generated by hiPSC-derived myogenic progenitors (single lineage muscles), to complex isogenic neuromuscular constructs containing vascular networks derived using hiPSC-derived myogenic, neural and vascular progenitors (bi-, tri- and tetra-lineage muscles). Moreover, the presence of PAX7+ (albeit less controllable than other cell types; figure 3C) increases the number of simultaneous isogenic cell lineages to five, potentially enabling studies of complex cell interactions in the human muscle stem cell niche in a quasi-vivo setup.

Derivation of cell lineages from hiPSCs relies on validated protocols. For skeletal myogenic cells, two different methods have been used: a transgene-based method [56] and a transgene-free method [60], although we do not foresee major hurdles in using alternative methods [55,57–59,61,62]. We recommend using transgene-based hiPSC myogenic differentiation if the specific research question/application does not require the faithful recapitulation of myogenic specification events (e.g. studies of phenotype in myotubes rather than in muscle stem/progenitor cells). Also, if complex multi-lineage constructs need to be developed, as cell culture requirements for non-myogenic cells (e.g. neural or vascular lineages) might interfere with small molecule-based myogenic differentiation of hiPSCs.

Once hiPSC derivatives have been characterised (details in the respective protocols [39,40,56,60,63]), 3D constructs can be generated using a platform originally developed for cardiac tissue engineering and

subsequently validated by our group for skeletal muscle engineering[1,20]. After polymerisation, the fibrin-Matrigel™ hydrogels appear soft and translucent (figure 2A). Over the following days, changes in the hydrogel texture occur and it becomes more opaque and thinner and shorter as the cells remodel the matrix. Constructs should be handled with care at any stage of differentiation. For maximal preservation of tissue architecture (e.g., myofibril alignment), it is recommended to fix the hydrogels *in situ*, i.e., without removing them from the PDMS post racks. Depending on the specific research question or application, 3D constructs can be analysed at different time points with lineage specific markers detected using one or multiple techniques as detailed in figure 3. We recommend assessment following a minimum of 7 days in differentiating conditions to enable formation of sarcomeric structures in myofibres and organisation of the neuromuscular and vascular cells. Longer time points (e.g., > 12 days) are required to detect contraction upon stimulation. Contractility data can be converted into force values using the formula and method detailed in Osaki *et al.*[32].

We have validated our platform using a library of over 10 healthy donor and patient-specific hiPSCs from different muscle diseases (e.g., Duchenne, limb-girdle 2D and LMNA-related muscular dystrophies). This method is also remarkably versatile in terms of applications: from tissue replacement[1] (figure 5C-H) to disease modelling (figure 4) and therapy screening (figure 5). Using this platform, we successfully modelled the dysmorphic myonuclear phenotype of skeletal muscle laminopathies, unravelling novel objective readouts (i.e., nuclear elongation) previously undetected in conventional monolayer cultures and currently being used to screen potential drug candidates in our laboratory. Regardless of the genotype and at variance with the flattened morphology of cell nuclei identifiable in monolayer cultures, rendering of confocal images will clearly show that nuclei in artificial muscles appear three-dimensional.

Artificial muscles can also be used to screen advanced therapies such as gene therapy vectors and cell therapies. We provide here proof of principle evidence of their use to monitor expression of a non-viral vector introduced in the myogenic cells prior to their combination with biomaterials in 3D (figure 5A) as well as of viral vectors directly delivered to the already assembled 3D muscles (figure 5B). We focused on AAVs as they are the most used and promising neuromuscular gene therapy vectors (e.g., onasemnogene abeparvovec for spinal muscular atrophy), using a simple fluorescent-based readout to show expression dynamics of different vector concentrations upon transduction of artificial muscle constructs. Transgene production can easily be monitored at the whole muscle level using fluorescent stereo microscopy and then confirmed with higher resolution microscopy in live cells or upon fixation of the tissue using specific antibodies (e.g., anti-GFP; Figure 5B). Depending on the AAV serotype and MOI, transgene expression in 3D muscle can be observed as early as 3 days post-transduction. Another personalised advanced therapy that can be assessed and optimised with this platform is muscle cell therapy: we provide here proof-of-principle assessment of migration of myogenic cells[68] within isogenic 3D muscles (with both derived from the same hiPSC source), providing a unique strategy to assess myogenic cell transplantation in a

quasi vivo environment to optimise future skeletal muscle-directed cell therapies (figure 5C). Finally, transgene expression can also be detected with similar modalities upon ex vivo explant of mouse muscles implanted with artificial muscles (figure 5D-H). Of note, muscle function experiments in immunodeficient mice subjected to volumetric muscle injury suggested better integration and rescue of function by tri-lineage artificial muscles (myofibers + ECs +PCs) than more conventional single lineage hydrogels[1].

DATA AVAILABILITY

The majority of the raw data reported in this paper have been deposited in Mendeley Data and are available at: https://doi.org/10.17632/d826fxhr3b.1 linked to the original publication. New data and images present in this manuscript will be deposited in Mendeley Data.

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COMPETING INTERESTS

FST has received speaker and consultancy honoraria from Takeda, Sanofi Genzyme and Aleph Farms (via UCL Consultants). All other authors declare no competing interests.

ETHICS DECLARATION

Work with human cells was performed under approval of the National Health Service (NHS) Health Research Authority Research Ethics Committee (reference No. 13/LO/1826) and Integrated Research Application System (IRAS) project (ID No. 141100).

AUTHOR CONTRIBUTIONS

LP, MK and FST wrote the paper, assembled the figures, and performed the protocolisation and optimisation of all the steps listed. LP performed laminopathy disease modelling experiments and analysis. MK performed calcium experiments and advanced imaging. MK and SS performed AAV experiments with support from JRC. VML performed contractility and calcium experiments with support from ASB. SD and SWC performed DYS-HAC and cell migration experiments, respectively. SMM and SS developed the 3D skeletal muscle platform with FST and discussed results. NK derived neural progenitor cells. EN performed work on immortalised human myoblasts provided by AB. PSZ co-supervised LP with FST during his PhD and contributed to his funding, and reviewed manuscript drafts. FST provided funding, coordinated the work, discussed and analysed results, wrote and reviewed manuscript drafts with all co-authors and finalised the paper.

Figures

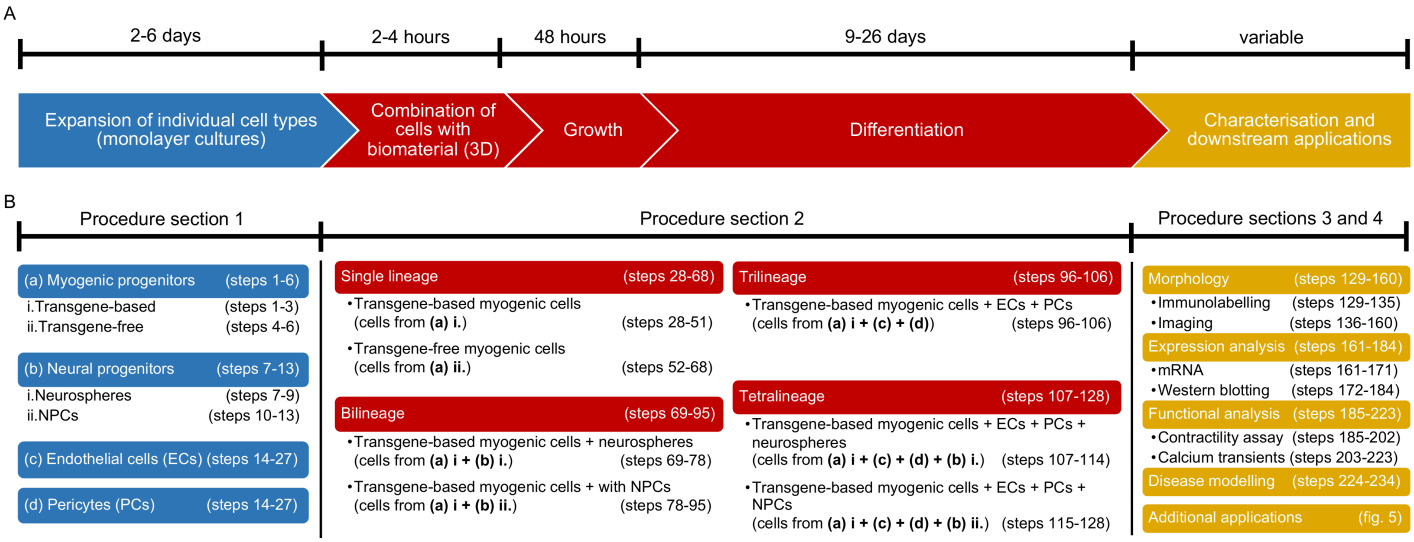

Figure 1

Workflow to generate and use 3D skeletal muscle constructs using hiPSC-derived progenitors. (A) Timeline of making 3D muscle constructs, detailing key stages of the protocol. **(B)** The three major stages of the procedure with corresponding steps (in brackets). The first stage (blue) entails the

monolayer expansion of the hiPSC derivatives from the various protocols/sources depending on the type of constructs required. The second stage (red) is making the single, bi-, tri- or tetra-lineage muscle constructs including polymerisation, growth and differentiation. The final stage (yellow) details downstream applications using 3D muscle constructs.

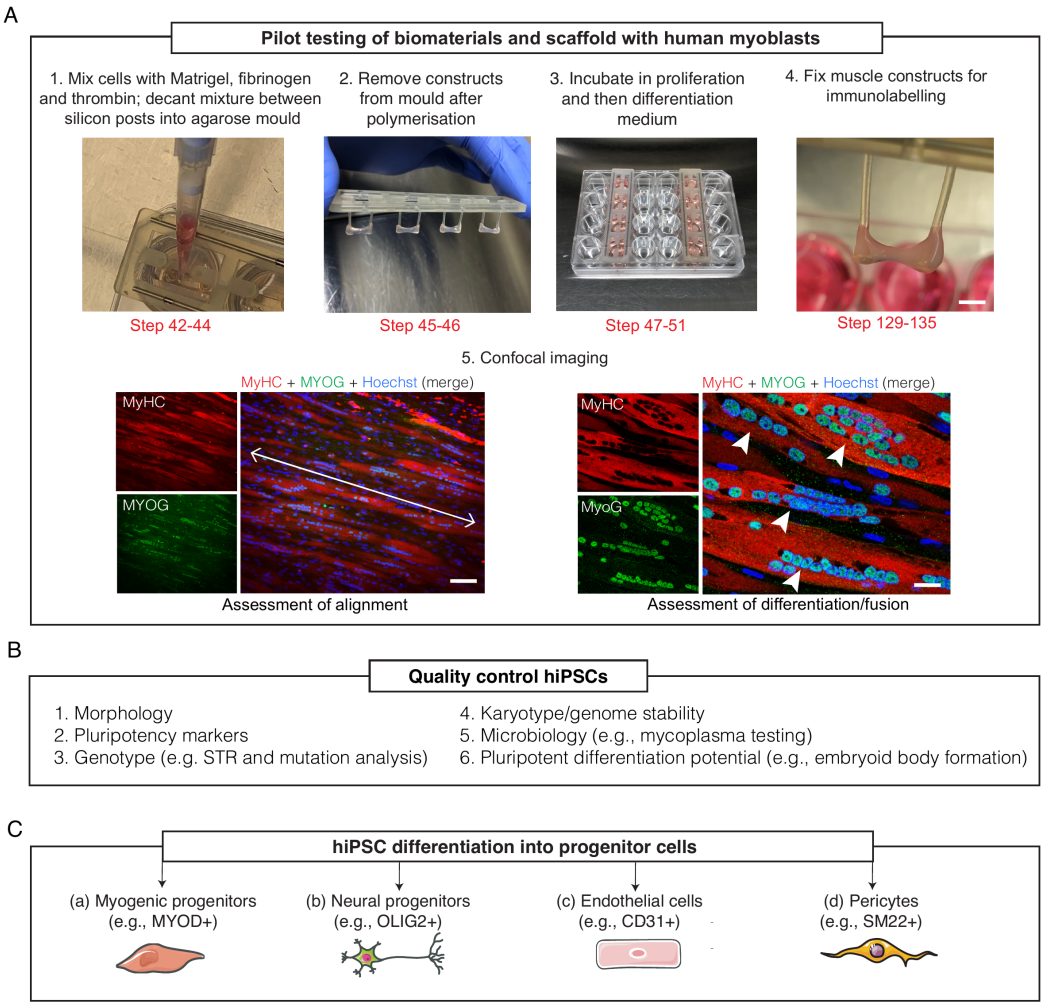

Figure 2

Pilot testing and quality control for biomaterials and cells. (A) Top images (1-4): Pilot testing of biomaterials (fibrinogen, thrombin and Matrigel™) and scaffold using tissue derived myoblasts prior to differentiating hiPSC derivatives in 3D muscle constructs. After generating the cell and biomaterials mixture as per the calculation sheet, supplementary table 1. (1-5) Key steps of the protocol showing polymerisation, culturing of constructs and macro morphology at the end of differentiation. Bottom panels (4): Immunolabelling for sarcomeric myosin heavy chain (MyHC, red) and the early differentiation marker Myogenin (MyoG, green) with Hoechst counterstain is used to assess the degree of myogenic differentiation. 1. Alignment of myoblast-derived myotubes in 3D along the passive tension axis, highlighted by white arrows; 2. Differentiation and accretion of further myoblasts fusing into larger multinucleated skeletal myotubes (arrowheads) after 10 days in culture. **(B)** Examples of common quality controls for hiPSCs (1 to 6) prior to use in 3D muscle constructs. **(C)** Schematic simplification of the hiPSC derivatives used in this protocol with examples of key markers to check their identify prior to use in 3D constructs (e.g. (a) MyoD for hiPSC-derived myogenic progenitors, (b) OLIG2 for neural progenitors, (c) CD31 for endothelial cells and (d) SM2 for pericytes (produced using Servier Medical Art-smart.servier.com). Scale bars: (A) Top right image 3 mm, bottom left image 100 µm, bottom right image 25 µm.

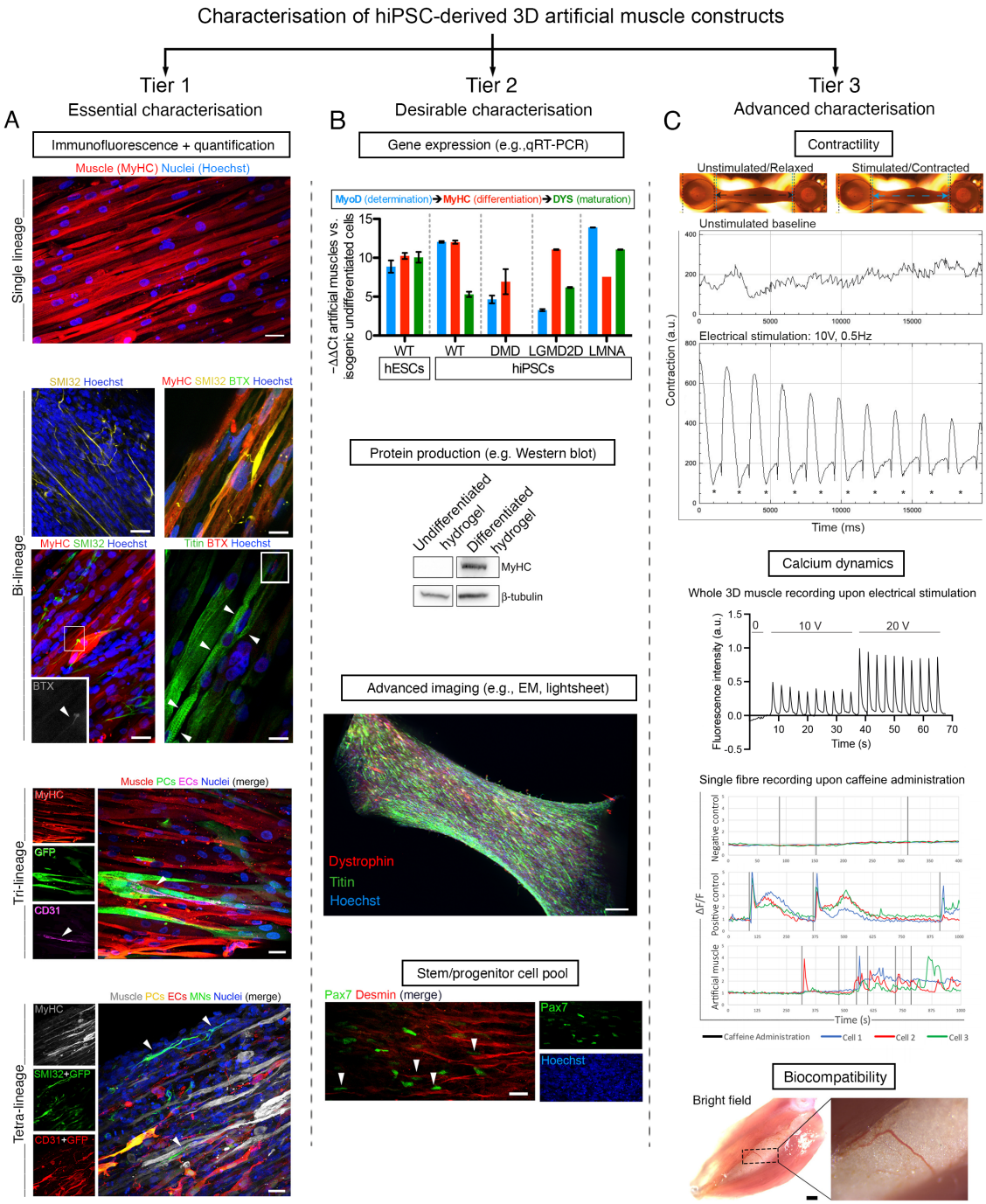

Figure 3

Characterisation of hiPSC-derived 3D artificial muscles. **(A)** Tier 1: Essential characterization of 3D muscles (immunolabelling and quantification). Confocal microscopy images of wholemount immunolabelled 3D muscles of increasing cellular complexity, from single (top) to tetra-lineage (bottom). Bi-linege panels show examples of: SMI-32+ axons inside the constructs (top left image), alpha-bungarotoxin (BTX)+ patches in proximity of myofibres and SMI32+ axons (top right and

bottom left images, highlighted by arrowhead); bottom left image shows a myofiber with sarcomeric TITIN+ striations (arrowheads) and BTX+ acetylcholine receptors (red signal in white box). Tri-lineage panel shows immunolabeling of a multilineage construct containing CD31+ endothelial cells (ECs) juxtaposed to GFP+ pericytes (PCs; arrowhead) and MyHC+ myofibers. (**B)** Tier 2: Desirable characterization of 3D muscles (gene expression, protein production, advanced imaging and assessment of stem cell-associated markers). Upper graph: qRT-PCR analysis for myogenic markers of single-lineage 3D muscles comparing expression levels of *MYOD*, *myosin heavy chain* (*MyHC*) and *DYSTROPHIN* (DYS: absent in DMD artificial muscles). Values normalised on *GAPDH*; ddCt is calculated on the corresponding expression values of undifferentiated cells. Central panels (top to bottom): western blot for MyHC (250 kDa) in undifferentiated cells and single-lineage hiPSC-derived 3D muscle. beta-tubulin: loading control (50 kDa); Light-sheet microscopy image of single-lineage artificial skeletal muscle showing Hoechst (blue) TITIN (green) and DYSTROPHIN (red) distribution; Bottom panel: immunofluorescence showing PAX7+ nuclei adjacent to DESMIN+ myofibres differentiated from hiPSCs in 3D for 14 days (transgene-free method). **(C)** Advanced characterization. From top to bottom: Contractility: Representative images of 3D muscles recorded from the top before and after stimulation (relaxed and contracted state highlighted by black and blue arrows, respectively); traces showing contractile response to electrical stimulation of 3D muscles generated from DMD hiPSC-derived myogenic cells at day 17 of differentiation; contractions were induced by electrical stimulation at 10 V and 0.5 Hz (*stimulus). Arbitrary units (a.u.) values represent the displacement of the scaffold's pillars over time (ms; shown in associated images); baseline (unstimulated) trace was obtained recording the muscle without electrical stimulation; traces are generated and analysed using ImageJ Muscle Motion plugin. Central panels: calcium dynamics in muscle constructs loaded with the fluorescent Ca2+ indicator Fluo-4AM upon upon electrical stimulation (10 V and 20 V at 0.3 Hz) on whole muscles or upon caffeine administration (10 mM) and recording of single myofibres. Bottom panel - Biocompatibility: stereoscopic images of a freshly explanted tibialis anterior muscle 1 month after implantation of an hiPSC-derived 3D muscle in an NSG immunodeficient mouse subjected to a volumetric muscle injury showing blood vessels crossing the implant. Images in: (A) single-, bottom two bi-, tri- and tetra-lineage pictures; (B) gene expression, protein production and stem cell pool panels; (C) Single fibre calcium dynamics and Biocompatibility panels from Maffioletti SM *et al.* https://doi.org/10.1016/j.celrep.2018.03.091 (CC BY 4.0). Scale bars: (A) 25 μm; (B) top: 500 μm; middle: 25 μm; (C) 1.5 mm.

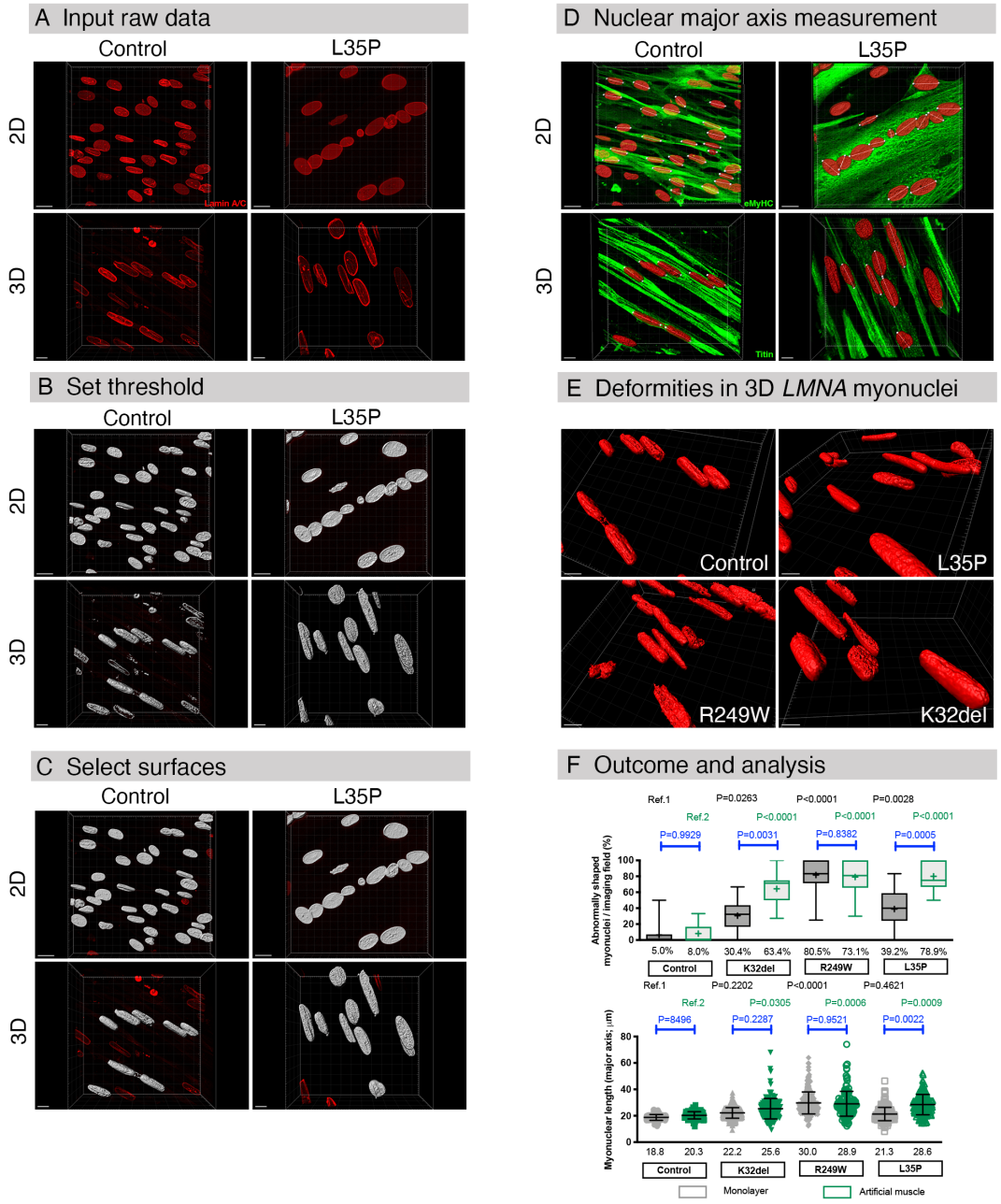

Figure 4

Modelling muscular dystrophies caused by abnormal nuclear envelope using hiPSC-derived 3D muscles: stepwise 3D nuclear reconstruction of hiPSC-derived healthy control and LMNA-mutant skeletal myotubes in monolayer vs. 3D artificial muscles. (A) Raw confocal images of healthy control and LMNA L35P mutant 2D and 3D skeletal muscle cultures inputted in Imaris®. **(B)** After clicking on to “Add new surface” from the left-hand menu to begin the reconstruction, the threshold is selected based upon

fluorescent intensity. **(C)** Undesired objects detected in (B) can be removed by selecting specific surfaces. **(D)** After executing the creation steps, the final reconstruction is completed and myofibres can be visualised by ticking the specific box under the “Display adjustment” tab. The extremities of the myonuclei can be set using the “Add new measurement point” tool. The distance between the two points is outputted automatically and measurements can be exported in an Excel spreadsheet through the “Statistics” tab (see Supplementary Information for more details). **(E)** High magnification of reconstructed myonuclei of healthy control and LMNA mutant 3D artificial muscles. **(F)** Analysis showing proportion of abnormal myonuclei (upper panel) and nuclear major axis length (bottom panel) in conventional monolayer cultures (grey boxes and whiskers/points) and 3D artificial muscles (green boxes and whiskers/points). Statistics, upper graph: two-way ANOVA with Tukey's post-hoc comparisons for comparisons to control, and Sidak's for comparisons within lines on the average of three repeats. Data are shown as box plots of proportion of abnormal nuclei per imaging field from all three repeats; whiskers: minimum and maximum values, +: average of all imaging fields. Values shown on graph: average of three repeats used for statistical analysis. P values are displayed at the top of the graph. Bottom graph: two-way ANOVA with Tukey's post-hoc comparisons to control, and Sidak's for comparisons within lines on the average of three repeats. Data are shown as a scatter plot comprised of all values from three repeats combined. Bars: mean and standard deviation. Nuclei were assessed to be part of a myotube based on location within multinucleated MyHC- or TITIN-positive structures. Scale bars: 20 μm for 2D monolayer cultures images (A-D); 10 μm for all 3D artificial muscles images (A-D) and nuclear deformities examples in LMNA constructs (E). Graphs in (F) and some images adapted from Steele-Stallard *et al.* https://doi.org/10.3389/fphys.2018.01332 (CC BY).

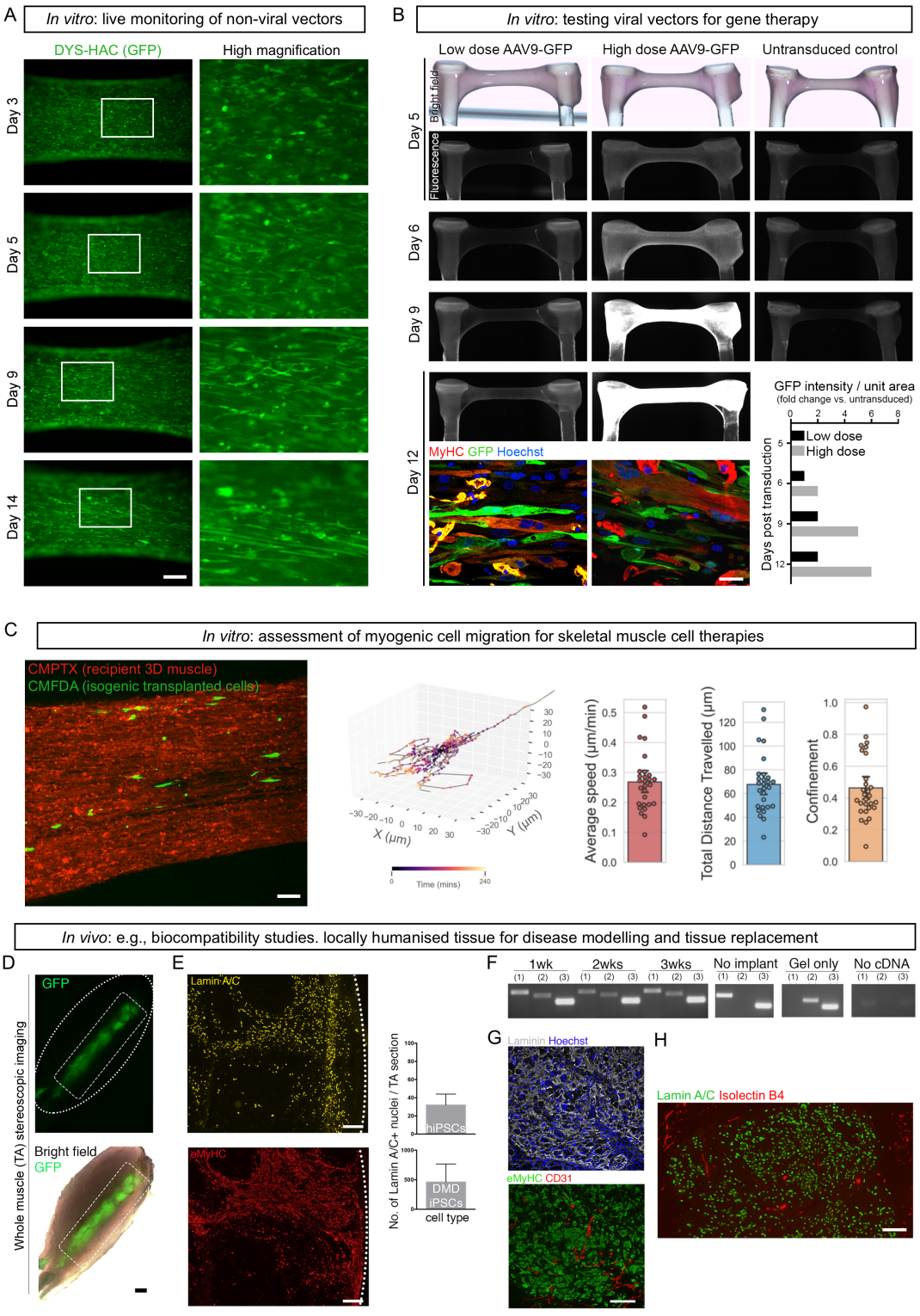

Figure 5

Examples of *in vitro* and *in vivo* applications of hiPSC-derived 3D artificial muscles: testing of gene therapy vectors and biocompatibility studies. (A) Live monitoring time-course of transgene expression from a non-integrating, non-viral vector in 3D hiPSC-derived artificial muscles. DMD hiPSCs engineered with a human artificial chromosome containing the full human *dystrophin* genetic locus and a GFP (DYS-HAC) transgene were differentiated into inducible myogenic progenitors and then assembled in 3D

hydrogels which were sequentially imaged over 2 weeks to monitor GFP expression and formation of myotubes in culture. **(B)** Proof-of-principle example of application of hiPSC-derived 3D muscles for testing and monitoring expression of transgenes expressed by adeno associated viral vectors (AAVs) for possible use in muscle gene therapies. Time-course of untransduced control and AAV9-transduced muscle constructs at low (MOI 1000) and high vector dose (MOI 100,000): brightfield and fluorescence stereomicroscopic imaging showing progressive increase in GFP fluorescence in live-imaged 3D constructs. Bottom immunofluorescence panels highlight AAV-driven GFP production in myosin heavy chain (MyHC) positive multinucleated myotubes (confocal imaging, maximum intensity projection; Hoechst: nuclei). Bottom right graph quantifies GFP intensity in transduced muscles over time vs. untransduced controls. **(C)** Live imaging of myogenic cell migration using isogenic 3D muscles. Left image: maximum intensity projection of CMFDA-stained hiPSC-derived myogenic cells on isogenic hiPSC-derived CMPTX+ artificial muscles. Central graph: trajectory plots displaying individual tracks of CMFDA+ cells after correcting for movement of the 3D muscles. Right graphs: bar plots displaying quantification of migration parameters of CMFDA+ cells including velocity, total distance travelled and confinement. **(D-H)** Biocompatibility *in vivo*: implantation, engraftment and vascularization of hiPSC- derived artificial muscles. **(D)** Stereoscopic pictures of a tibialis anterior (TA) muscle 3 weeks after implantation of a GFP+ hiPSC-derived artificial muscle in NSG mice. **(E)** Immunofluorescence panel showing engrafted human LAMIN A/C+ nuclei in transverse sections of the TA muscle shown in (C) and quantified in (E), alongside staining of a serial section with embryonic MyHC showing regenerating myofibers in the area engrafted with human nuclei. **(F)** RT-PCR confirming engraftment of human artificial muscles by showing expression of the muscle-specific *alpha sarcoglycan* gene (*SGCA*; 2) in the transplanted mouse TA at different time points. Control muscles only express mouse *Sgca* (1) whereas human artificial muscles cultured *in vitro* only express human *SGCA. GAPDH*: loading control (3). **(G)** Immunofluorescence images showing extracellular matrix (laminin), newly generated myofibres (eMyHC) and endothelial cells (CD31) within the implanted artificial muscle in TA transverse sections. **(H)** Immunofluorescence image showing systemically delivered, 594-conjugated fluorescent isolectin B4 (red) labelling endothelial cells within the implanted human artificial muscle, demonstrating presence of functional blood vessels withing the implant (LAMIN A/C: human nuclei). Images in panels (C-H) adapted from Maffioletti SM *et al.* https://doi.org/10.1016/j.celrep.2018.03.091 (CC BY 4.0). Scale bars: (A) 500 μm; (B) 30 μm; (C) 100 μm; (D) 1.5 mm; (E) 200 μm; (G, H) 100 μm.

Supplementary Files

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