Scalable human skeletal myocytes by opti-ox reprogramming of iPSCs for the study of muscle biology, neuromuscular junction and metabolic disorders

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Roisin Nicoll[1], Will Bernard[1], Imbisaat Geti[1], Tonya Frolov[1], Patrick Rericha[1], Tuzer Kalkan[1], Michael D'Angelo[1], Giacomo Borsari[1], Dougall Norris[2], Daniel Fazakerley[2], Shushant Jain[3], Michael Duchen[4], Gabriel Esteban Valdebenito[4], Farah Patell-Socha[1], Thomas Moreau[1], Mark Kotter[1]

[1] bit.bio, The Dorothy Hodgkin Building, Babraham Research Campus, Cambridge, CB22 3FH, UK. [2] Metabolic Research Laboratories, Wellcome-MRC Institute of Metabolic Science, University of Cambridge, UK. [3] Charles River Laboratories, Leiden, The Netherlands, 2333CR. [4] Division of Biosciences, University College London, UK. Corresponding author's e-mail: roisin.nicoll@bit.bio

Abstract

Skeletal myocytes play roles in a number of biological processes ranging from limb movement to the regulation of nutritional homeostasis and are implicated in the pathophysiology of a variety of diseases such as muscular dystrophies and metabolic disorders. There is a pressing need for reliable models of mature human skeletal muscle to permit investigations into physiological and disease mechanisms, and to facilitate the generation of new therapeutics. While human induced pluripotent stem cells (hiPSCs) offer a promising starting material for skeletal muscle cells, their broad use has been hampered by difficult to reproduce and complex differentiation protocols. We have developed an optimised inducible system (opti-ox™) that enables tightly controlled expression of transcription factors, improving cellular reprogramming approaches for the differentiation of hiPSCs. Through targeting of genomic safe harbour loci, we used opti-ox to achieve homogenous, inducible expression of the myogenic regulator MYOD1[1,2]. MYOD1 induction leads to shutdown of the core pluripotency network and activation of key myogenic factors including myosin heavy chain. Cryopreserved ioSkeletal Myocytes homogeneously express the key proteins of the myofilaments Desmin, Dystrophin and Titin, and form striated and multinucleated myocytes that contract in response to acetylcholine by 10 days post-revival. The skeletal muscle phenotype and culture homogeneity have been further analysed by RNA sequencing to provide in depth characterization. Importantly, ioSkeletal Myocytes produce a highly pure Myosin Heavy Chain positive population of cells within only 4 days of thawing and are amenable to high-throughput screening pipelines. Demonstrating the value of the ioSkeletal Myocytes for muscle functional studies, Airyscan z-series stacks reveal structural proteins in a striate structure that contract in response to electrical stimulation and increased extracellular potassium levels. Critically for metabolic studies, robust expression of the insulin-regulated glucose transporter GLUT4 is also detected. The scalability and robustness of opti-ox reprogramming and ease of use of the ready to culture ioSkeletal Myocytes provide a unique hiPSC based model for research of muscle biology, including disease modelling and high throughput applications.

1. Precise reprogramming of iPSCs into defined human cell types

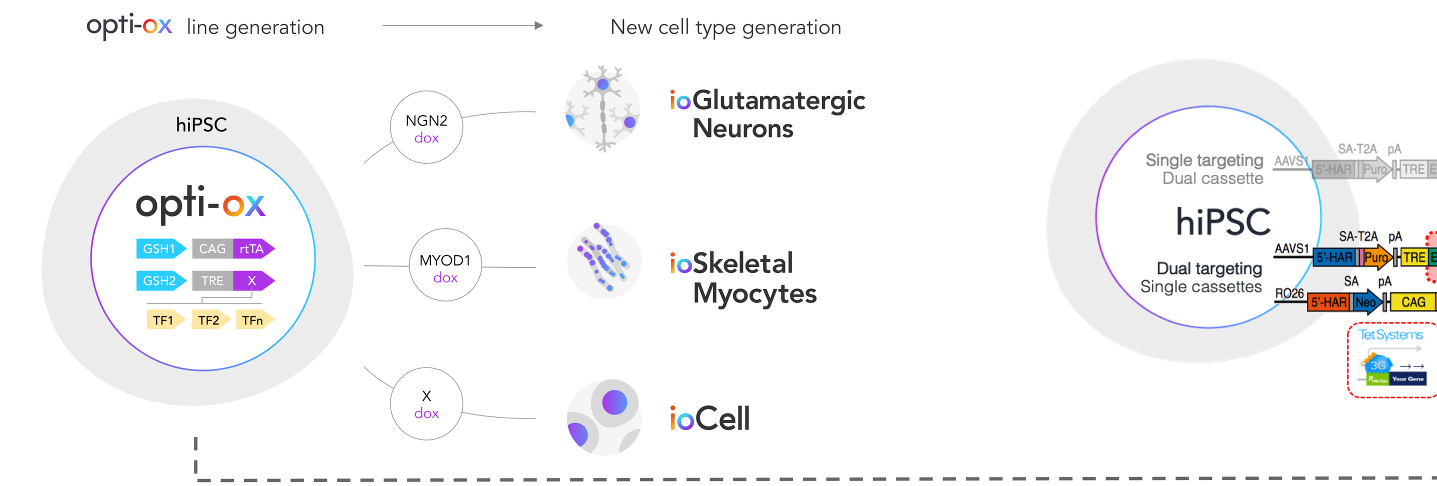
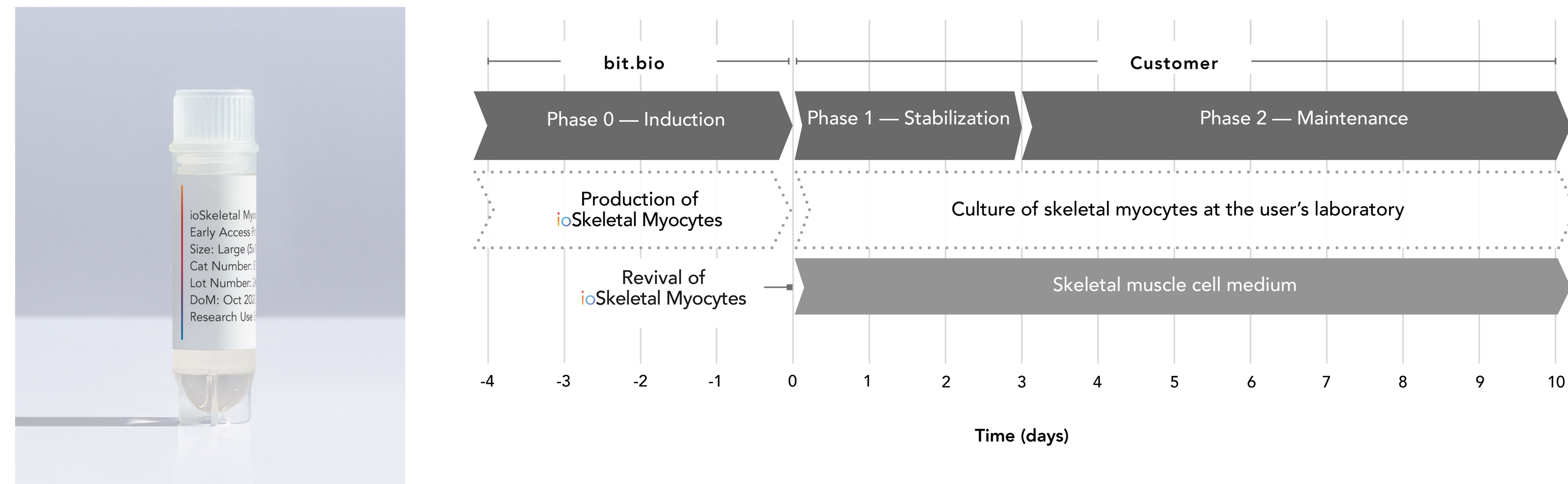


Figure 1. opti-ox technology for the optimal cellular reprogramming of human iPSCs into defined human cell types. opti-ox dual cassette Tet- ON system ensures tightly controlled and homogeneous expression of reprogramming transcription factors by preventing silencing of the inducible expression cassette after genetic engineering of hiPSCs[2].

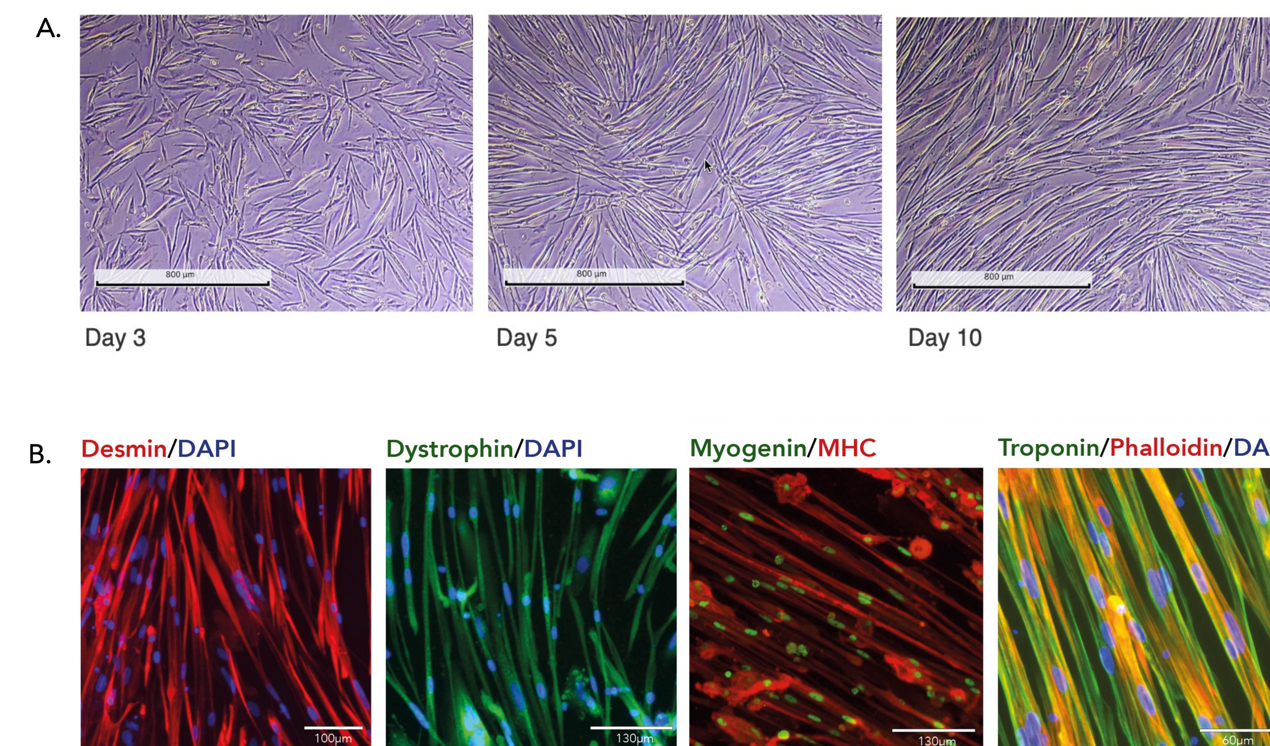
2. Human muscle cells ready for experimentation within days

Figure 2. ioSkeletal Myocytes are derived from hiPSCs by MYOD1 driven opti-ox reprogramming and arrive ready to plate. Cells are delivered in a cryopreserved format and are programmed to rapidly mature upon revival in the recommended media. The protocol for the generation of these cells is a three-phase process: 1. Induction (carried out at bit.bio); 2. Stabilization for 3 days with Doxycycline; 3. Maintenance during which the skeletal myocytes mature.



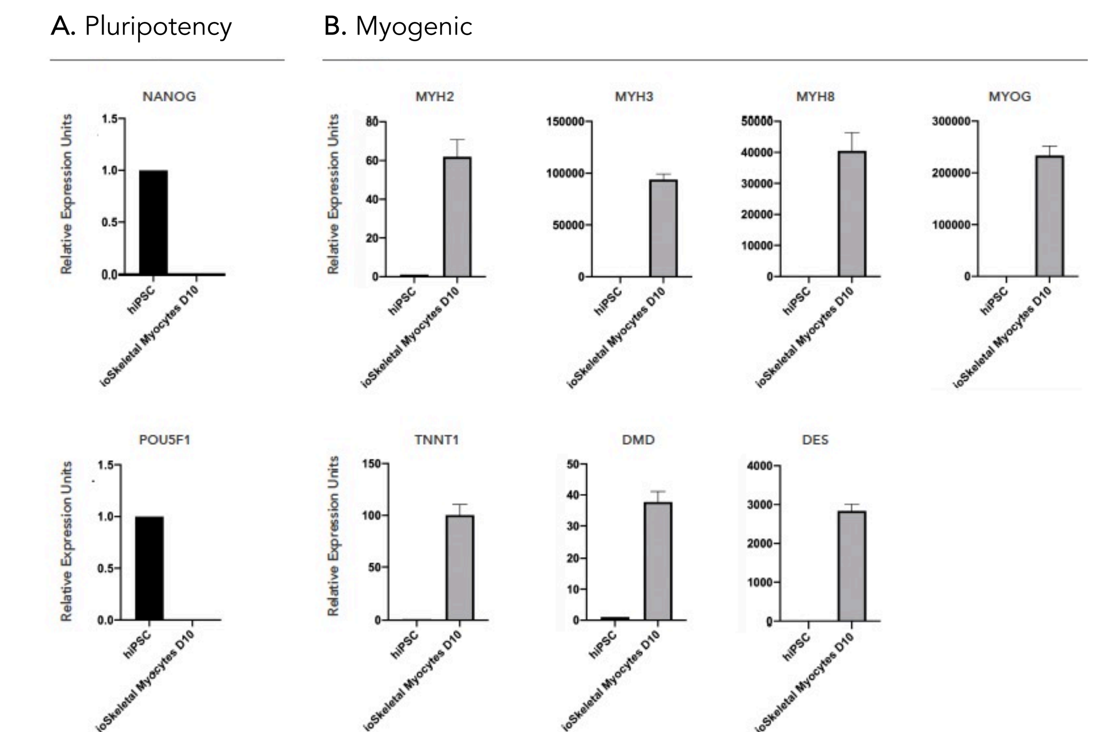
3. ioSkeletal Skeletal Myocytes form contractile, elongated fibres over 10 days and express mature myogenic markers

Figure 3: Characterization of ioSkeletal Myocytes. (A) ioSkeletal Myocytes after revival over the course of the first 10 days of culture. Day 1 to 10 post-thawing; 4X magnification; scale bar: 800µm. **(B)** Immunofluorescence staining at day 10 post revival demonstrates robust expression of components of the contractile apparatus such as Desmin, Dystrophin, and Myosin Heavy Chain (MHC), along with the muscle transcription factor Myogenin. Cells also demonstrate expression of Troponin with visible striated fibres, and multinucleation.



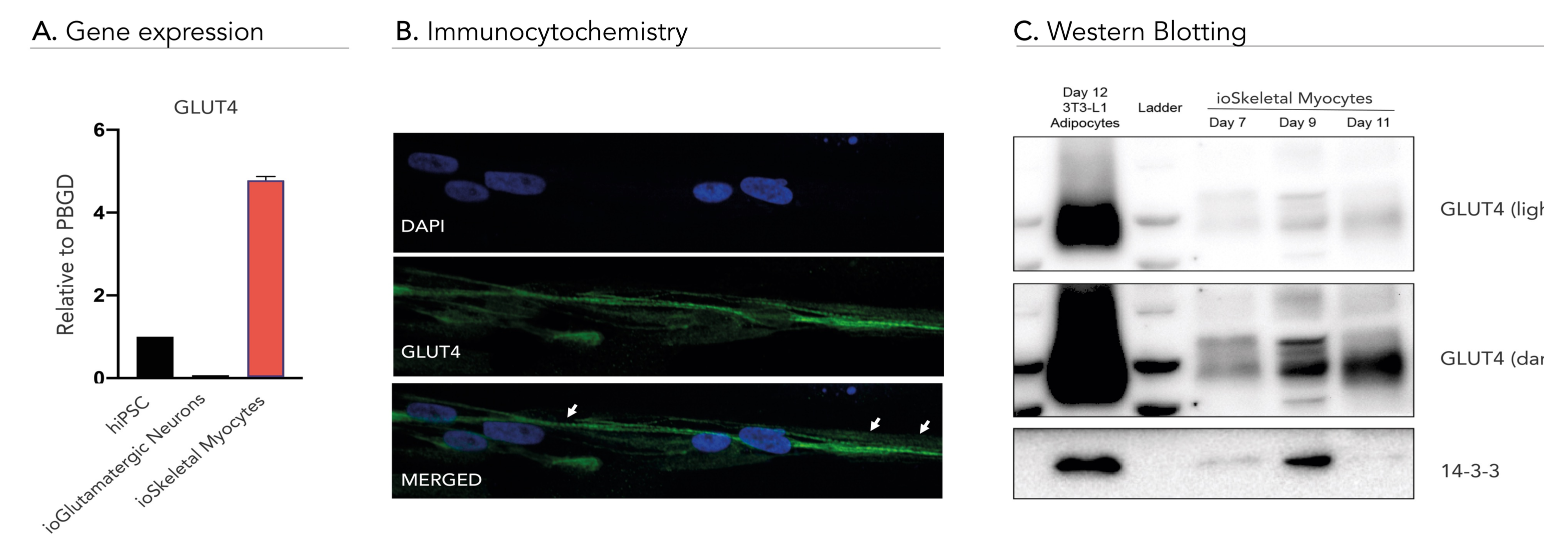
4. Cells demonstrate gene expression of key myogenic markers following reprogramming

Figure 4: ioSkeletal Myocytes gene expression. Following reprogramming, ioSkeletal Myocytes downregulate expression of the pluripotency genes (A), whilst demonstrating robust expression of key myogenic markers) (B). Gene expression levels assessed by RT-qPCR (data expressed relative to the parental hiPSC, normalised to HMBS). Data represents Day 10 post-revival samples; n=7 biological replicates.



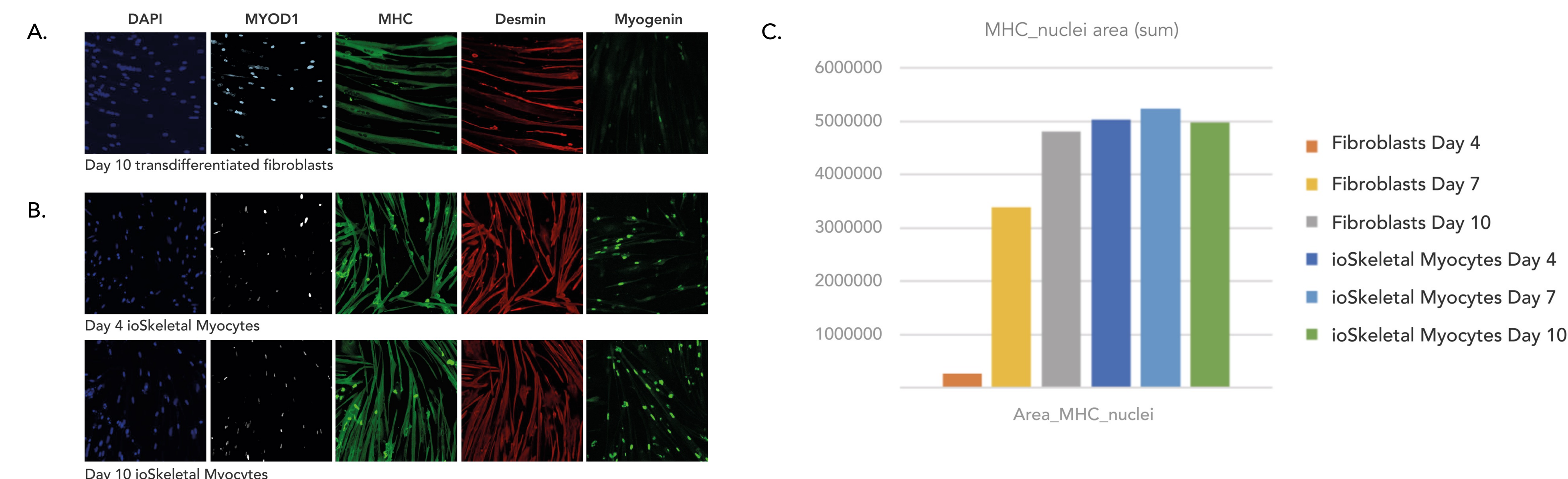
5. Cells express the insulin regulated glucose transporter GLUT4, critical for metabolic studies

Figure 5: Data demonstrates expression of the insulin regulated glucose transporter GLUT4, suggesting that ioSkeletal Myocytes provide a unique human cell model for metabolic research. (A) RT-qPCR at Day 10 post-revival demonstrating expression of GLUT4 in the Skeletal Myocytes, compared to undifferentiated hiPSCs and ioGlutamatergic Neurons. **(B)** Immunocytochemistry at Day 7 post-revival demonstrates expression of GLUT4 in peri-nuclear regions, and striations, in the ioSkeletal Myocytes[3]. **(C)** Western blotting of differentiated 3T3-L1 adipocytes and maturing ioSkeletal Myocytes demonstrates GLUT4 expression in a time-dependent manner[3].



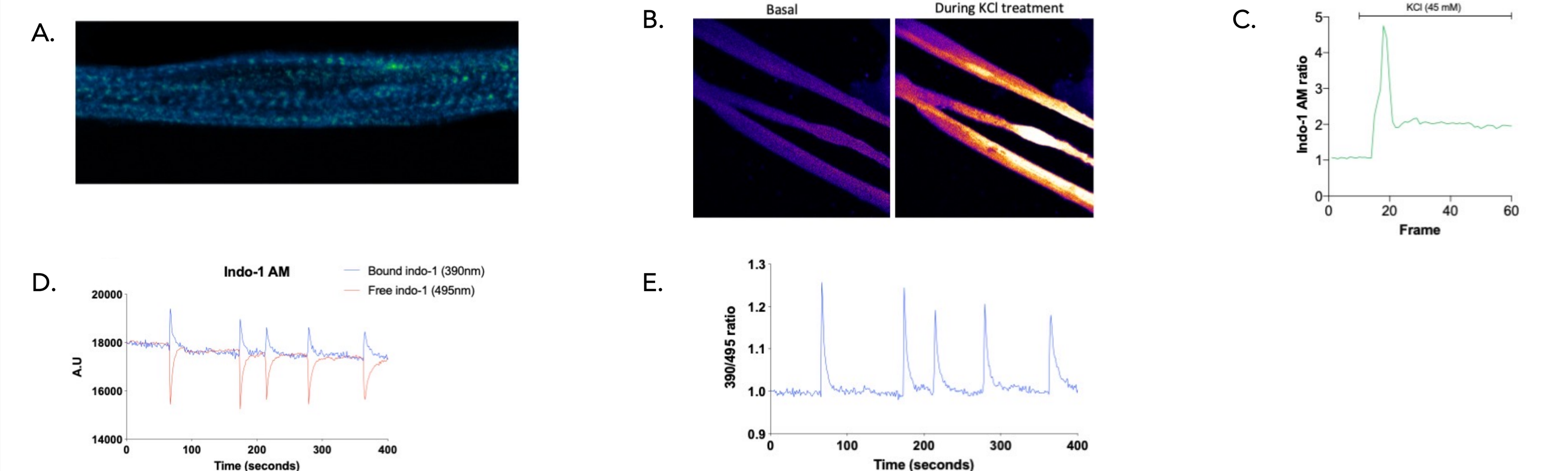
6. Myocytes are suitable for phenotypic based HTS

Figure 6: ioSkeletal Myocytes generate myocytes within as little as 4 days post-revival with a high degree of MHC+ cells[4]. (A) Human fibroblasts were transduced with lentiviral vectors allowing inducible over-expression of MYOD1 to transdifferentiate them to myocytes in approximately 10 days. Transdifferentiated myotubes were stained for multiple myotube markers to assess the purity and degree of multi-nucleation. **(B)** iPSCs stably and inducibly expressing MYOD1 using opti-ox technology (ioSkeletal Myocytes) can generate myocytes within as little as 4 days from revival with a high-degree of MHC+ cells (>95% purity), suitable for phenotypic based high throughput screens. **(C)** Comparable total area of MHC positive cells are generated between ioSkeletal Myocytes and transdifferentiated fibroblasts. These results suggest that the ioSkeletal Myocytes are amenable to HTS.



7. Striated myocytes contract in response to electrical stimulation and increased extracellular potassium levels

Figure 7: Changes in intracellular Calcium (Ca^{2+}) content after chemical and electrical stimulation[5]. (A) Immunofluorescence staining of alpha-actinin in ioSkeletal Myocytes revealing robust expression of sarcomere structures. **(B)** Representative images of ioSkeletal Myocytes incubated with Indo-1 AM 5 uM and 0.02% Pluronic F127. Cells were excited at 355nm on a UV-visible confocal, and emission measured simultaneously at 390 and 495nm. **(C)** Changes in Indo-1 AM ratio shows Ca^{2+} influx induced by 45 mM KCl. **(D-E)** Electrical stimulation (2 Hz, 6 v, 2 ms) shows the repetitive ability of ioSkeletal Myocytes to induce calcium Ca^{2+} release and sequestration.



1. Davis et al., Cell, 1987. **2.** Pawlowski M, et al., Stem Cell Reports, 2017. **3.** Dougall Norris & Daniel Fazakerley, Wellcome-MRC Institute of Metabolic Science. **4.** Shushant Jain et al, Charles River Laboratories. **5.** Gabriel E. Valdebenito & Michael R. Duchen, 2021. Department of Cell and Development Biology and Conortium for Mitochondrial Research, UCL.