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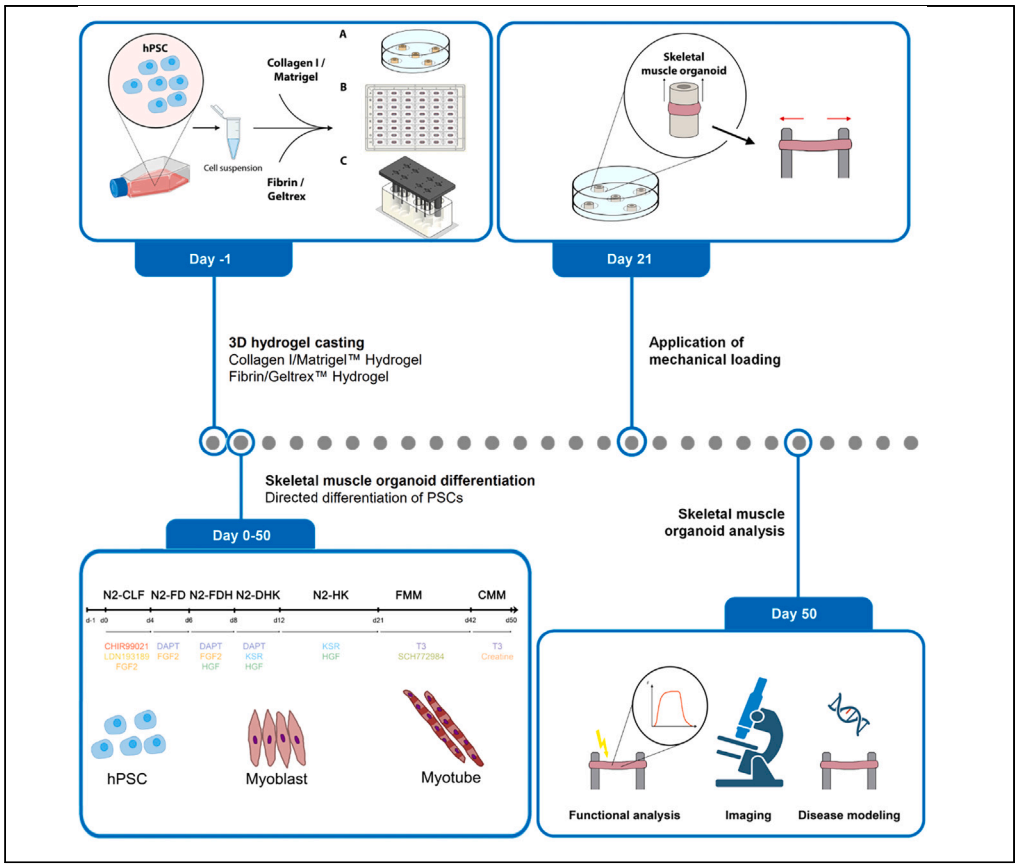
Protocol to develop force-generating human skeletal muscle organoids



Force generation is an essential property of skeletal muscle models *in vitro*. We describe a versatile 1-step procedure to direct undifferentiated human pluripotent stem cells (PSCs) into contractile skeletal muscle organoids (SMOs). Our protocol provides detailed steps for 3D casting of PSCs using either collagen-I/Matrigel- or fibrin/Geltrex-based hydrogels, SMO differentiation, and application of different culture platforms for mechanical loading and contractility analysis. The SMO model may be particularly useful to study human muscle development and developmental skeletal muscle disorders *in vitro*.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Multicellular skeletal muscle organoids from human pluripotent stem cells

1-step generation of contractile skeletal muscle in a 3D hydrogel

Applicable to different hydrogel compositions and culture platforms

Recapitulates human muscle development and disease

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Protocol to develop force-generating human skeletal muscle organoids

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SUMMARY

Force generation is an essential property of skeletal muscle models *in vitro*. We describe a versatile 1-step procedure to direct undifferentiated human pluripotent stem cells (PSCs) into contractile skeletal muscle organoids (SMOs). Our protocol provides detailed steps for 3D casting of PSCs using either collagen-I/Matrigel- or fibrin/Geltrex-based hydrogels, SMO differentiation, and application of different culture platforms for mechanical loading and contractility analysis. The SMO model may be particularly useful to study human muscle development and developmental skeletal muscle disorders *in vitro*.

For complete details on the use and execution of this protocol, please refer to Shahriyari et al.[1]

BEFORE YOU BEGIN

This protocol describes the generation of skeletal muscle from human pluripotent stem cells using 3D hydrogels. We provide two protocols for different types of hydrogel (Collagen I- and Fibrin-based) that can be applied interchangeably. In both types of hydrogel, contractile muscle is generated by directing pluripotent stem cells into muscle cells via a serum-free differentiation protocol. In addition, organotypic non-muscle cells that support tissue generation are formed. Shape and size of the resulting tissue may vary according to culture platform. Three different examples are given (Figure 1), but the protocol may be applicable to any other culture platform to generate tissue engineered muscle.

The generation of skeletal muscle organoids (SMO) requires the user to be well trained in feeder-free culture of human pluripotent stem cells.

Institutional permissions

Before executing this protocol, the user must ensure that work with human pluripotent stem cells has been approved by the respective Institution. Informed consent and ethical approval by the University Medical Center Göttingen was obtained for use of human iPSC lines (10/9/15).

Preparation of cell culture reagents

Timing: 6 h

1. Prepare cell culture reagents under aseptic conditions in a biosafety cabinet.
2. $CHIR_{99021}$ (10 mM):
   a. Reconstitute $CHIR_{99021}$ in DMSO to obtain a 10 mM stock solution.
   b. Aliquot (50 μL) and store at −20°C for up to one year.
   c. Once thawed, keep at 4°C for up to one week.
3. LDN_{193189} (10 mM):
   a. Reconstitute LDN_{193189} in DMSO to obtain a 10 mM stock solution.
   b. Aliquot (10 μL) and store at −20°C for up to one year.
   c. Once thawed, dilute 1:10 in DMSO to a 1 mM working concentration and keep at 4°C for up to one week.
4. FGF-2 (10 μg/mL):
   a. Dissolve FGF-2 in PBS containing 0.1% recombinant albumin to obtain a 10 μg/mL stock solution.
   b. Aliquot (100 μL) and store at −20°C for up to one year.
   c. Once thawed, keep at 4°C for up to one week.
5. DAPT (20 mM):
   a. Reconstitute DAPT in DMSO to obtain a 20 mM stock solution.
   b. Aliquot (50 μL) and store at −20°C for up to one year.
   c. Once thawed, keep at 4°C for up to one week.
6. HGF (10 μg/mL):
   a. Dissolve HGF in PBS containing 0.1% recombinant albumin to obtain a 10 μg/mL stock solution.
   b. Aliquot (50 μL) and store at −20°C for up to one year.
   c. Once thawed, keep at 4°C for up to one week.
7. Y27632 (10 mM):
   a. Reconstitute Y27632 in DMSO to obtain a 10 mM stock solution.
   b. Aliquot (50 μL) and store at −20°C for up to one year.
   c. Once thawed, keep at 4°C for up to one week.
8. Triiodo-L-thyronine (61.45 μM):
   a. Dissolve 1 mg Triiodo-L-thyronine (T3) in 1 mL of 1 N NaOH and dilute into 24 mL basal maturation medium to obtain a 61.45 μM stock solution.
   b. Sterile filter (0.2 μm), aliquot (depending on experiment design between 50 and 200 μL) and store at −20°C for up to one year.

   Note: A fresh T3 aliquot should be used for every medium change. Please prepare single-use aliquots and discard after thawing.

9. SCH 772984 (1 mM):
   a. Reconstitute SCH 772984 in DMSO to obtain a 1 mM stock solution.
   b. Aliquot (50 μL) and store at −20°C for up to one year.
   c. Once thawed, keep at 4°C for up to one week.
10. Creatine monohydrate (10 mM):
   a. Dissolve Creatine monohydrate in basal maturation medium to obtain a 10 mM stock solution. It will take approximately 15 min for creatine powder to fully dissolve. Shaking gently while keeping the solution warm (37°C) is necessary to dissolve the powder properly.
   b. Aliquot (depending on experiment design between 2.5 and 5 mL) and store at −20°C for up to one year.

   Note: Once thawed, first shake the solution gently to dissolve the precipitate. Please prepare single-use aliquots and discard after thawing.

11. N-2 supplement (100×):
   a. Thaw the N-2 supplement protected from light. Aliquot (500 μL) and store at −20° for up to 18 month.

b. Thaw N-2 supplement aliquots at room temperature protected from light and immediately add it to the medium as described in the tables below.
c. Store N-2 supplement aliquots at 4°C after thawing for maximum of 1 week protected from light.

12. B-27 supplement (50×):
    a. Thaw the B-27 supplement protected from light. Aliquot (1 mL) and store at −20°C for up to one year.
    b. Thaw B-27 supplement aliquots at room temperature and immediately add it to the medium as described in the tables below.
    c. Store B-27 supplement aliquots at 4°C after thawing for maximum of 1 week protected from light.
13. Matrigel:
    a. Thaw Matrigel (7–10 mg/mL total protein) on ice at 4°C for 16–24 h.
    b. Prepare 250 μL aliquots into 50 mL falcon tubes using ice-cold pipette tips and tubes, and directly transfer aliquots to −20°C freezer.
    c. Store aliquots at −20°C for up to one year.
14. Thrombin (100 U/mL):
    a. Dissolve thrombin from human plasma in sterile water to obtain a 100 U/mL stock solution by gently swirling.
    b. Aliquot (10 μL) and store at −20°C.

Note: Please prepare single-use aliquots and discard after thawing.

15. Fibrinogen (10 mg/mL):
    a. Dissolve fibrinogen in 0.9% NaCl solution to obtain a 10 mg/mL stock solution. It will take approximately 5 min at 37°C for fibrinogen to completely dissolve.
    b. Quickly spin down the mixture before incubating at 37°C to dissolve fibrinogen powder properly.
    c. Sterile filter (0.2 μm) fibrinogen solution before use.

Note: Please prepare fresh single-use aliquots before each experiment and avoid vortexing or any harsh mixing.

16. 6-aminocaproic acid (ACA):
    a. Dissolve ACA in distilled water to obtain a 50 mg/mL solution.
    b. Sterile filter (0.2 μm) solution before use.
    c. Aliquot (5 mL) and store at −20°C.
17. 10× DMEM:
    a. Add 1.34 g DMEM powder in 10 mL distilled water. Let dissolve under constant shaking for at least 1 h at 37°C, sterile filter (0.2 μm).
18. 2× DMEM:
    a. Mix 2 mL of 10× DMEM with 8 mL distilled water, sterile filter (0.2 μm) and keep for max. 2 weeks at 4°C.
19. NaOH (0.1 N):
    a. Dilute 1 N NaOH 1:10 in distilled water; sterile filter (0.2 μm) and keep at 4°C.
20. Collagen (Acid Solubilized Telo Collagen):
    a. We recommend to obtain a collagen stock solution of 5–7 mg/mL.
    b. Before aliquoting, rotate the stock bottle very gently to ensure homogenous distribution.
    c. Aliquot collagen on ice (10 mL) and keep the aliquots at 4°C.

Note: Care must be taken to store and handle collagen at 4°C; collagen must never be frozen or handled at room temperature.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
6-Aminocaproic acid (ACA)	Sigma-Aldrich	Cat. #A2504
B-27 supplement	Thermo Fisher Scientific	Cat. #17504044
CHIR99021	ReproCELL	Cat. #04-0004
Collagen (acid solubilized telo collagen)	Collagen Solutions LLC	Cat. #FS22024
Creatine monohydrate	Sigma-Aldrich	Cat. #C3630
DAPT	Tocris	Cat. #2634
DMEM, low glucose, GlutaMAX supplement, pyruvate	Thermo Fisher Scientific	Cat. #10567014
DMEM, powder, low glucose, pyruvate	Thermo Fisher Scientific	Cat. #31600083
DMSO	Sigma-Aldrich	Cat. #D2650
Geltrex	Thermo Fisher Scientific	Cat. #1413202
FGF-2	PeproTech	Cat. #AF-100-18B
Fibrinogen	Sigma-Aldrich	Cat. #F8630
HGF	PeproTech	Cat. #100-39
Human serum albumin, recombinant	Sigma-Aldrich	Cat. #A9731
Knockout serum replacement	Thermo Fisher Scientific	Cat. #10828028
LDN193189	ReproCELL	Cat. #04-0074
Matrigel (growth factor reduced)	Corning	Cat. #354230
MEM non-essential amino acid solution	Thermo Fisher Scientific	Cat. #11140035
2-Mercaptoethanol	Thermo Fisher Scientific	Cat. #31350010
N-2 supplement	Thermo Fisher Scientific	Cat. #17502048
NaCl	Carl Roth	Cat. #7647-14-5
NaOH 1 mol/L (1 N)	Carl Roth	Cat. #K021.1
PBS 1×	Thermo Fisher Scientific	Cat. #14190094
Penicillin/Streptomycin	Thermo Fisher Scientific	Cat. #15140122
SCH 772984	Cayman Chemical	Cat. #19166
StemMACS iPS-Brew XF medium	Miltenyi Biotec	Cat. #130104368
Thrombin	Sigma-Aldrich	Cat. #T6884
Triiodo-L-thyronine (T3)	Sigma-Aldrich	Cat. #T2877
Versene solution (0.48 mM EDTA)	Thermo Fisher Scientific	Cat. #15040066
Y27632 (ROCK inhibitor)	ReproCELL	Cat. #04-0012-10
Experimental models: Cell lines		
Human wild-type iPSC line TC-1133	RUCDR	Baghbaderani et al., 2016
TC1133-ACTN2-Citrine	UMG	Härtter et al., submitted
Other		
myrPlate Uniform	myriamed	TM5-MED

MATERIALS AND EQUIPMENT

Culture platforms for SMO formation

We have generated SMO utilizing different platform technologies. One approach would be to generate custom-made silicone molds to produce ring-shaped SMO that can be transferred to stainless steel holders for maturation.[1,2,5] Alternatively, a commercially available tissue culture plate for 48 SMO in parallel,[3] and a custom-made chamber to allow high-power imaging[4] will work similarly well (Figure 1).

Pluripotency medium for SMO generation		
Reagent	Final concentration	Amount
StemMACS iPS-Brew XF medium	N/A	45 mL
Y27632	5 μM	25 μL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
FGF-2	10 ng/mL	50 µL
Knockout serum replacement	10%	5 mL
Total	N/A	50.1 mL

Note: The Pluripotency medium for SMO generation should be prepared fresh before use.

Note: Add the reagents and the supplement after warming up the StemMACS iPS-Brew XF medium to 37°C.

Basal differentiation medium (N-2 medium)		
Reagent	Final concentration	Amount
DMEM low glucose (1 g/L), GlutaMAX supplement and pyruvate	N/A	485 mL
Penicillin-Streptomycin	1%	5 mL
N-2 Supplement (100×)	1%	5 mL
MEM non-essential amino acid solution (100×)	1%	5 mL
Total	N/A	500 mL

Note: Store N-2 medium at 4°C for maximum 2 weeks.

Note: The pH of the N-2 medium should not become too basic, which may be traced using the phenol red indicator in the medium (a fuchsia-red color of the medium should be avoided). Aliquot the N-2 medium according to the volume you need for each day medium change and avoid opening the stock bottle of N-2 medium every day.

N2-CLF medium (day 0, 1, 2 and 3 of SMO differentiation)		
Reagent	Final concentration	Amount
N-2 medium	N/A	15 mL
C: CHIR99021	10 µM	15 µL
L: LDN193189	0.5 µM	7.5 µL
F: FGF-2	10 ng/mL	15 µL
Total	N/A	15 mL

Note: The N2-FCL medium should be prepared fresh before use.

Note: Add the small molecules and growth factors after warming up the basal differentiation medium to 37°C.

N2-FD medium (day 4 and 5 of SMO differentiation)		
Reagent	Final concentration	Amount
N-2 medium	N/A	15 mL
F: FGF-2	20 ng/mL	30 µL
D: DAPT	10 µM	7.5 µL
Total	N/A	15 mL

Note: The N2-FD medium should be prepared fresh before use.

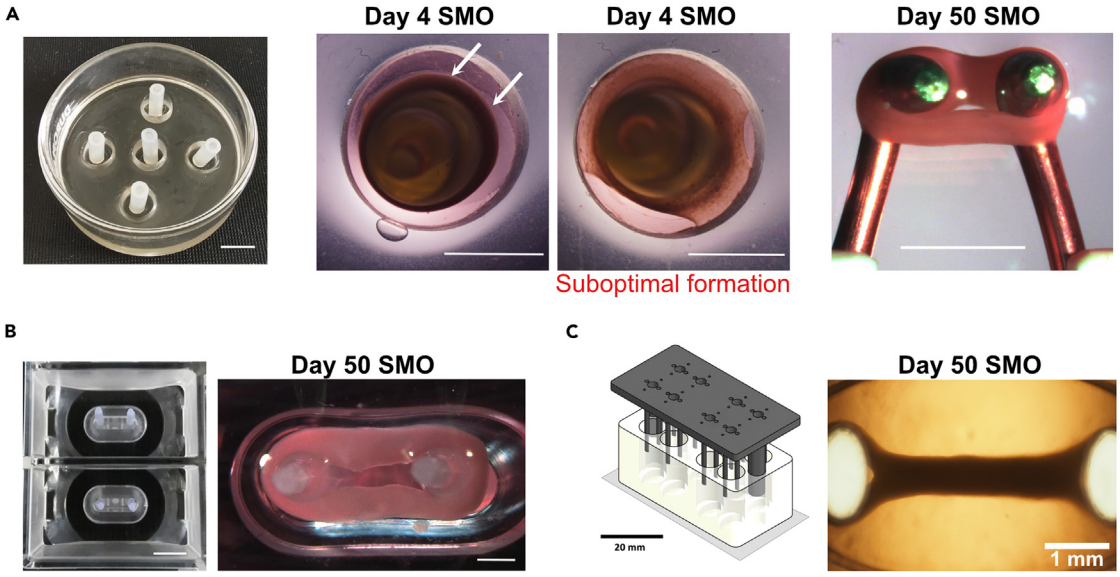

Figure 1. Generation of skeletal muscle organoids (SMO) in multiple technological platforms

(A) Silicone molds (re-usable) to generate ring-shaped SMO (250 μL hydrogel volume); Scale bar: 10 mm. SMO hydrogel will compact around a central silicone tube (Day 4 SMO). By day 4, tissue compaction should be completed (arrows in Day 4 SMO), delay in compaction may indicate suboptimal cell differentiation. The ring-shaped SMO is then transferred to stainless steel holders for maturation under mechanical load[2]; Scale bar: 5 mm.

(B) Single use plate format to generate 48 ring-shaped SMO in parallel (180 μL hydrogel volume).[3] Scale bar: 5 mm, 1 mm (Day 50 SMO).

(C) Casting chamber for 8 strip-shaped SMO in parallel (25 μL hydrogel volume).[4] Scale bars: 20 mm; 1 mm.

Note: Add the small molecules and growth factors after warming up the differentiation basal medium to 37°C.

N2-FDH medium (day 6 and 7 of SMO differentiation)		
Reagent	Final concentration	Amount
N-2 medium	N/A	15 mL
F: FGF-2	20 ng/mL	30 μL
D: DAPT	10 μM	7.5 μL
H: HGF	10 ng/mL	15 μL
Total	**N/A**	**15.1 mL**

Note: The N2-FHD medium should be prepared fresh before use.

Note: Add the small molecules and growth factors after warming up the differentiation basal medium to 37°C.

N2-DHK medium (day 8, 9, 10 and 11 of SMO differentiation)		
Reagent	Final concentration	Amount
N-2 medium	N/A	13.5 mL
D: DAPT	10 μM	7.5 μL
H: HGF	10 ng/mL	15 μL
K: Knockout serum replacement	10%	1.5 mL
2-Mercaptoethanol	0.1 mM	30 μL
Total	**N/A**	**15.1 mL**

Note: The N2-HDK medium should be prepared fresh before use.

Note: Add the small molecules and growth factors after warming up the differentiation basal medium to 37°C.

N2-HK medium (day 12 to 21 of SMO differentiation)		
Reagent	Final concentration	Amount
N-2 medium	N/A	13.5 mL
H: HGF	10 ng/mL	15 μL
K: Knockout serum replacement	10%	1.5 mL
2-Mercaptoethanol	0.1 mM	30 μL
Total	**N/A**	**15 mL**

Note: The N2-HK medium should be prepared fresh before use.

Note: Add the growth factor and the knockout serum after warming up the differentiation basal medium to 37°C.

Basal maturation medium		
Reagent	Final concentration	Amount
DMEM low glucose (1 g/L), GlutaMAX supplement and pyruvate	N/A	480 mL
Penicillin-Streptomycin	1%	5 mL
N-2 Supplement (100×)	1%	5 mL
B-27 Supplement (50×)	2%	10 mL
Total	**N/A**	**500 mL**

Note: Store Basal maturation medium at 4°C for maximum 2 weeks.

Note: The pH of the Basal maturation medium should not become too basic, which may be traced using the phenol red indicator in the medium (a fuchsia-red color of the medium should be avoided). Aliquot Basal maturation medium according the volume you need for each day medium change and avoid opening the stock bottle every day.

Fusion maturation medium (FMM; week 1, 2 and 3 of maturation)		
Reagent	Final concentration	Amount
Basal maturation medium	N/A	15 mL
Triiodo-L-thyronine (T3)	0.1 μM	24.4 μL
SCH 772984	1 μM	15 μL
Total	**N/A**	**15 mL**

Note: The Fusion maturation medium should be prepared fresh before use.

Note: Add T3 and SCH 772984 after warming up the basal maturation medium to 37°C.

Creatine maturation medium (CMM; week 4 onwards)		
Reagent	Final concentration	Amount
Basal maturation medium	N/A	13.5 mL
Triiodo-L-thyronine (T3)	0.1 μM	24.4 μL
Creatine monohydrate	1 mM	1.5 mL
Total	**N/A**	**15 mL**

Note: The Creatine maturation medium should be prepared fresh before use.

Note: Add T3 and creatine after warming up the basal maturation medium to 37°C.

STEP-BY-STEP METHOD DETAILS

Preparation of human pluripotent stem cells (PSC) for SMO generation

Timing: 20 min

This step describes the preparation of a cell suspension for SMO generation from human pluripotent stem cell cultures.

Note: All the volumes are considered for a T75 cell culture flask. For other culture plate surface areas volumes should be adjusted accordingly.

1. Maintain human PSC lines on 1:120 Matrigel in phosphate-buffered saline-coated plates.
   a. Prepare Matrigel-coated plates (T75 flask).
      i. Resuspend a frozen Matrigel aliquot in 29.75 mL 4°C PBS to obtain the working dilution of 1:120.
      ii. Add 6 mL of diluted Matrigel per T75 flask and ensure that the entire surface is covered.
      iii. Incubate at 37°C for at least 30 min before plating the cells.

   Note: Coated plates can be stored at 4°C for up to 2 weeks but should not be used if the Matrigel-PBS solution has dried up (i.e. not fully coating the culture vessel surface).

   Note: Wrapping the cell culture dish rim with parafilm is recommended to prevent evaporation of Matrigel-PBS solution.

2. Culture human PSC lines in StemMACS iPS-Brew XF (Miltenyi Biotec) at 37°C and 5% CO_2.
3. Dissociate the cultures when they reach a confluency of 70%–80%.

   Note: You should consider that from a T75 flask with optimal confluency approximately 8 million cells may be obtained which is enough for 2 mL of SMO hydrogel to be generated.

4. Warm up the StemMACS iPS-Brew XF medium and Versene solution to 37°C.
5. Add Rock inhibitor Y27632 to iPS-Brew XF medium to a final concentration of 5 μM.
6. Rinse the cell culture with 3 mL Versene solution, aspirate and incubate the culture in 5 mL Versene solution for 4–5 min at room temperature.
7. After approximately 4 min, observe the cells under the microscope to check if the cell culture is dissociated. Rounded detached cells should be observed.

   Note: If you do not see signs of dissociation tap the sides of the flask gently for several times and again look if the cells are detaching noticeably. If not, continue the incubation for another minute while you observe the cells under the microscope every 30 seconds.

8. To stop dissociation, add 10 mL of StemMACS iPS-Brew XF medium with 5 μM Rock inhibitor.
9. Continue with pipetting up and down, using a 10 mL pipet to make a single cell suspension.

   Note: Avoid excessive pipetting. Stop pipetting when there are no visible cell clumps.

10. Transfer the cell suspension to a 50 mL falcon tube.

Table 1. Composition of Collagen/Matrigel hydrogel master mix for SMO

Reagent	Final concentration	Amount
Acid soluble collagen type 1 (6.5 mg/mL)	0.92 mg/mL	142 μL
2× DMEM	N/A	142 μL
NaOH 0.1 N	N/A	27 μL
Matrigel	10% (v/v)	100 μL
Cell suspension	3.2×10 E^6/mL	589 μL
Total		**1000 μL**

11. To count the cells, take a sample from a homogenous cell suspension and use an automated cell counter or hemocytometer.

Note: Cell suspension can stay at room temperature while you count the cells.

⚠ CRITICAL: For an optimal SMO generation, the cell viability at this step should be above 80%.

12. Immediately after counting the cells, transfer the amount of cells for the desired volume of hydrogel (Tables 1 and 2) to another 50 mL falcon tube and centrifuge it at 300 × *g* for 4 min at room temperature.

Note: The input cell number/SMO may need to be optimized for different PSC lines since it will affect the efficiency of mesodermal induction. If tissue compaction is severely impaired by day 4 of differentiation or SMO do not contract (please see troubleshooting – problem 1 and 3) we recommend to test the lower (2.4 × 10E6 cells/1 mL master mix) and upper limits (4 x 10E6 cells/1 mL master mix) of input cells numbers first. Depending on the outcome and the growth rate of PSC the optimal concentration (3.2 x 10E6 cells/1 mL hydrogel) may be adapted in one or the other direction in smaller steps (0.2 × 10E6 cells/step).

13. Carefully aspirate the supernatant
    a. To continue with the Collagen I/Matrigel hydrogel method (steps 15–18, Table 1), resuspend the cell pellet in StemMACS iPS-Brew XF medium with 5 μM Rock inhibitor, 10 ng/mL FGF-2 and 10% Knock out serum replacement (Pluripotency medium) and place on ice.

Note: The volume for resuspension will depend on the amount of hydrogel to be generated (Table 1, e.g. 589 μL medium for a 1000 μL hydrogel with 3.2 × 10E6 cells).

    b. To continue with the Fibrin/Geltrex hydrogel method (steps 19–22, Table 2), leave the cell pellet on ice.
14. Immediately continue with preparation of hydrogel master mix for SMO generation.

Generation of SMO (collagen I/Matrigel hydrogel method)

Timing: 1.5 h

Table 2. Composition of Fibrinogen/Geltrex hydrogel master mix for SMO

Reagent	Final concentration	Amount
DMEM	40% (v/v)	400 μL
Fibrinogen (10 mg/mL)	40% (v/v)	400 μL
Geltrex	20% (v/v)	200 μL
Cell number	3.2×10 E^6/mL	N/A
Total		**1000 μL**

In this step, a final SMO hydrogel mixture of i) 0.92 mg/mL acid soluble collagen type 1 ii) concentrated 2× DMEM (0.27 g DMEM powder in 10 mL ddH_2O), iii) NaOH 0.1 N, iv) 10% v/v Matrigel and v) 3.2 x 10E6 iPSC/ml resuspended in StemMACS iPS-Brew XF medium with 5 μM Y27632, 10 ng/mL FGF-2 and 10% knockout serum replacement is prepared.

Note: Volumes of the other SMO components have to be adapted according to the volume of the collagen stock solution needed to obtain an optimal SMO collagen content. The amount of 2×DMEM should match the amount of collagen stock added. The NaOH amount may have to be adapted individually for optimal pH neutralization (see Critical point below).

15. Defrost a Matrigel aliquot with required volume on ice approximately 15 min before you start with preparation of hydrogel master mix.
16. Place the required reagents (Table 1) to prepare hydrogel master mix on ice.
    a. Place an additional 50 mL falcon tube on ice for preparation of the hydrogel.
    b. Take reagents back to 4°C storage immediately after you are done with casting
17. To prepare the SMO hydrogel, mix the components on ice in a 50 mL falcon tube and in the exact order listed in Table 1:

Note: Avoid holding the tube of each component in your hand and leave the tube on ice while pipetting the desired volume, especially for collagen and Matrigel, which solidify quickly when warming up.

    a. Add the Acid soluble collagen type 1.

Note: Collagen is very viscous. Pipet very slowly to avoid air bubble formation.

⚠ CRITICAL: Pipet the collagen at the bottom of the tube slowly and not on the side wall of the tube to avoid losses.

    b. Add the 2× DMEM serum-free medium on the collagen and mix by tapping the tube lightly to the side of the ice box or by pipetting gently without creating air bubbles.
    c. Add the NaOH 0.1 N and mix by tapping the tube lightly to the side of the ice box or by pipetting gently without creating air bubbles.

⚠ CRITICAL: pH neutralization of the acid soluble collagen must be confirmed by inspection of the phenol red indicator transition from yellow to pink (evaluated ~30 seconds after mixing). At this point, the condensation (solidification) of collagen will commence and must not be disrupted. Therefore, the following steps should be performed swiftly.

    d. Add Matrigel directly into the hydrogel and mix by swirling the tube very gently or by tapping the tube lightly to the side of the ice box.

Note: Do not pipette the hydrogel up and down at this stage.

    e. Add the cell suspension and mix by pipetting 3 times, but carefully to avoid air bubble formation and immediately continue with casting.
18. Cast SMO hydrogel using a 1 mL pipet tip. For an even distribution of the hydrogel pipet gradually and accurately while you move the pipette tip inside the receiving mold.

Note: Uneven distribution of the hydrogel will result in formation of a non-uniform SMO which may tear at the maturation step.

19. Carefully transfer the mold to the incubator and let the mixture gel for 1 h at 37°C.

20. Gently add 4 mL per mL of SMO hydrogel of 37°C StemMACS iPS-Brew XF medium with 5 μM Y27632, 10 ng/mL FGF-2 and 10% knockout serum replacement and return to the incubator.
21. Continue with step 28 for SMO generation.

Generation of SMO (Fibrin/Geltrex hydrogel method)

Timing: 1 h

This section provides another method to generate the SMO hydrogel as an alternative to steps 15–21.

Note: Do not resuspend the cell pellet during step 13, instead place the cell pellet directly on ice. Prepare the following hydrogel master mix during centrifugation to minimize the time for the cell pellet to sit on ice.

A final hydrogel mixture of i) 40% (v/v) DMEM, ii) 4 mg/mL Fibrinogen in 0.9% NaCl, iii) 20% (v/v) Geltrex, iv) 3.2 × 10^6 iPSC/mL and v) 0.5 U Thrombin per mg of Fibrinogen is prepared.

22. Defrost a Geltrex aliquot with required volume on ice approximately 15 min before you start with preparation of hydrogel master mix.
23. Place the required reagents (Table 2) to prepare hydrogel master mix on ice.
    a. Place an additional 15 mL falcon tube on ice for preparation of the hydrogel.
    b. Take reagents back to 4°C storage immediately after you are done with casting.
24. To prepare the SMO hydrogel, mix the components on ice in a 15 mL falcon tube and in the exact order listed in Table 2:

Note: Avoid holding the tube of each component in your hand and leave the tube on ice while pipetting the desired volume, especially for Geltrex, which solidifies very quickly when it gets warm.

    a. Add DMEM.
    b. Add Fibrinogen (10 mg/mL in 0.9% NaCl).
    c. Add Geltrex.

Note: Fibrinogen and especially Geltrex is very viscous. Pipet very slowly to avoid air bubble formation.

    d. Mix by swirling the tube very gently or by tapping the tube lightly to the side of the ice box.
    e. Loosen the cell pellet by carefully tapping and flicking the tube.
    f. Add the hydrogel mixture to the cells and mix by pipetting 5 times, but carefully to avoid air bubble formation.
    g. Add 0.5 U Thrombin per mg of Fibrinogen (20 μL of 100 U/mL stock per 1 mL of hydrogel), mix quickly by gently flicking the tube and immediately continue with casting.

Note: Always keep your hydrogel-cell mixture on ice and work quickly since Thrombin initiates the conversion of Fibrinogen to Fibrin clots.

25. Cast the SMO hydrogel evenly into the respective cell culture platform using an ice-cold pipet tip. For an even distribution of the hydrogel, pipet accurately while you move the pipet tip in a circular/ellipsoid motion.

Note: Uneven distribution of the hydrogel will result in formation of a non-uniform SMO, which will tear at the maturation step.

26. Carefully transfer the mold to the incubator and let the mixture gel for 3–5 min at 37°C.

Note: Also transfer the rest of the hydrogel-cell mixture to 37°C as a gelling control.

27. Gently add 4 mL per mL of hydrogel of 37°C StemMACS iPS-Brew XF medium with 5 μM Y27632, 10 ng/mL FGF-2, 1.5 mg/mL ACA and 10% knockout serum replacement to the side of each well and return to the incubator.

Note: Medium should be prepared freshly prior to use as described in material and equipment.

⚠ CRITICAL: For the Fibrin/Geltrex method 1.5 mg/mL ACA needs to be added to the media (i.e. 3 mL of 50 mg/mL ACA stock solution to 97 mL of medium) until day 21 of differentiation. After day 21 the ACA concentration is increased to 2 mg/mL (i.e. 4 mL of 50 mg/mL ACA stock solution to 96 mL of medium) during maturation to prevent matrix degradation.

Directed differentiation of SMO hydrogel

Timing: ≥50 days

In this step, the SMO hydrogel with pluripotent stem cells is differentiated into contractile muscle tissue.

28. On day 0 (24 h after casting the hydrogel), switch medium to N2-CLF (4 mL per mL of SMO hydrogel). Change daily on day 1, 2, and 3. Medium should be prepared freshly prior to use as described in material and equipment.

Note: Between 24 h after casting and first 4 days of differentiation, the SMO hydrogel should have compacted (Figure 1A).

29. On day 4 and 5, change the medium daily with 4 mL per mL of SMO hydrogel of N2-FD. Medium should be prepared freshly prior to use as described in material and equipment.
30. On day 6 and 7, change the medium daily with 4 mL per mL of SMO hydrogel of N2-FDH. Medium should be prepared freshly prior to use as described in material and equipment.
31. On day 8, 9, 10 and 11, change the medium daily with 4 mL per mL of SMO hydrogel of N2-DHK. Medium should be prepared freshly prior to use as described in material and equipment.
32. On day 12–20 change the medium every second day with 4 mL per mL of SMO hydrogel of N2-HK. Medium should be prepared freshly prior to use as described in material and equipment.
33. On day 21 SMOs should be subjected to mechanical loading either by growing SMOs between flexible poles (Figures 1B and 1C) or by actively transferring SMOs to stretch devices (Figure 1A).[5]
    a. Change medium to fusion maturation medium (FMM; ≥ 4 mL per mL of SMO hydrogel) for a total duration of 3 weeks with medium changes every other day.

Note: The use of SCH 772984 will facilitate myocyte fusion.[6]

34. After day 42, continue culture in creatine maturation medium (CMM) with medium changes every other day.

EXPECTED OUTCOMES

The protocol has been successfully applied to various platforms for muscle generation. Examples are a custom-made silicone mold to yield muscle rings that can be transferred to metal holders for

mechanical loading.[1,2,5] Alternatively, a commercially available tissue culture plate for 48 muscle tissues in parallel,[3] and a custom-made chamber to allow high-power imaging[4] may be used (Figures 1A–1C). Independent of the culture platform, a compact tissue should form within 4 days (Figure 1A) that will further compact until day 50 (Figures 1A–1C).

Muscle generation follows specific developmental stages, which can be monitored by prototypical gene expression (Figure 2A). In parallel with increasing expression of sarcomere genes like *ACTN2*, the formation and organization of *ACTN2*+ myocytes into elongated myofibers takes place during maturation between day 29 and day 50 (Figure 2B). Extending the culture period beyond day 50 is possible and will in fact further enhance maturation. Around day 29 of SMO development spontaneous contractions become visible. Independent of the platform, this protocol supports the generation of contractile SMO (Figure 2C). Depending on the platform technology, contractile force can be measured either by optical means facilitating higher-throughput analyses or by utilizing organ baths and force transducers for more comprehensive functional analysis (Figure 2C).

LIMITATIONS

While differentiation is very reproducible in SMO, it is a lengthy process and holding steps to monitor if tissue formation is proceeding correctly and to predict proper muscle formation may be needed. In addition, when running numerous different PSC lines, additional quality control measures may be required to compare functional output of SMO.

TROUBLESHOOTING

Problem 1

Tissue does not compact within 4 days (step 27).

Potential solution

- Usually due to the quality of the input iPSC. Make sure not to use over-confluent cells and optimize digestion to increase survival.
- Optimize the number of input cells/SMO for each PSC line and each culture platform. Lack of compaction typically requires to increase the input cell number/SMO. We recommend to test stepwise increases (0.2 × 10E6 cells per step) from 3.2 × 10E6 cells/1 mL master mix to 4 × 10E6 cells/1 mL master mix.
- Poor quality of the components of the hydrogel may also be the cause. Make sure that all reagents have not expired, have been stably stored and kept at 4°C.
- During preparation of collagen/Matrigel hydrogel, confirm that the phenol red indicator shows a color change form yellow to pink.
- Adding cold media to the culture device can adversely affect the process of SMO generation, especially during the first 4 days of differentiation. Make sure that your medium is warmed up to 37°C before use.

Problem 2

Tissue tears during culture (steps 32/33).

Potential solution

- Ensure homogenous cell distribution and homogeneous casting of the hydrogel.
- Avoid the generation of bubbles during hydrogel preparation by slow and careful pipetting.
- Make sure that hydrogel components (Collagen, Fibrinogen, Matrigel, Geltrex) are kept at 4°C. Premature gelation will prevent homogenous tissue formation.
- Directly after casting, ensure swift transfer to the incubator to allow hydrogel formation at 37°C for no less than 3 min (Fibrin/Geltrex hydrogel) or 60 min (Collagen/Matrigel hydrogel)

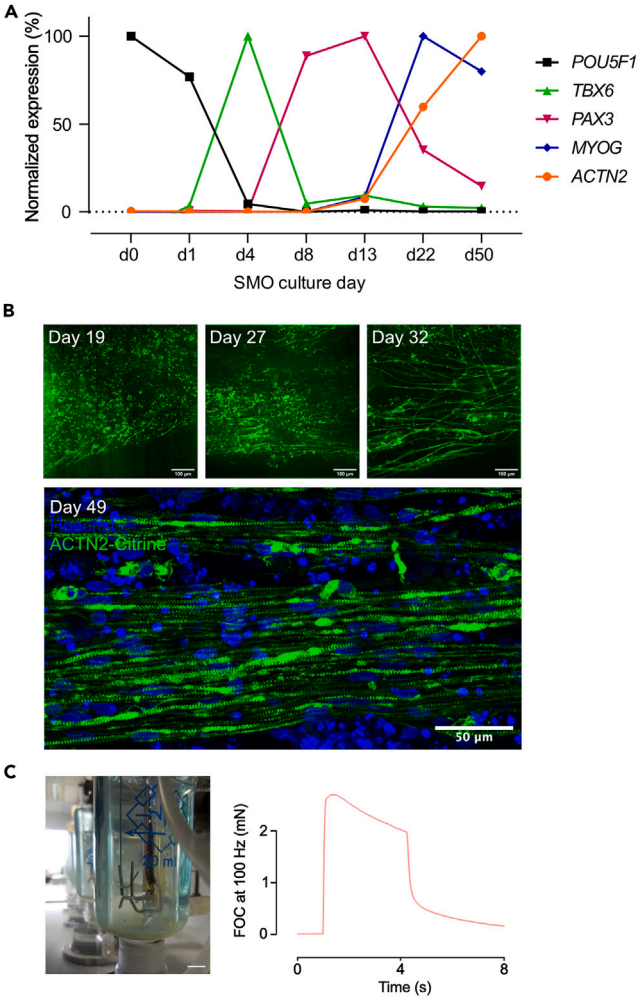

Figure 2. Muscle development in SMO

(A) Prototypical gene expression during SMO differentiation. Concurrent with loss of pluripotency gene expression (*POU5F1*), expression of paraxial mesoderm genes increases (*TBX6*), followed by expression genes indicative of somitogenesis (*PAX3*). Consecutively, expression of myogenic regulatory factors (like *MYOG*) indicates formation of muscle cells that mature with enhanced sarcomerogenesis (*ACTN2*).

(B) Visualization of muscle cells formation in SMO using a transgenic ACTN2-Citrine line.

(C) Force of contraction (FOC) of SMO can be measured in classical organ baths; Scale bar: 10 mm. Stimulation of SMO with 100 Hz induces tetanic contractions in SMO.

Problem 3

SMO do not contract.

Potential solution

- Monitor differentiation of the same cell line in 2D monolayer culture to ensure muscle cell formation.[1]
- Optimize paraxial mesoderm induction in the first 4 days of differentiation. To achieve an optimum paraxial mesoderm induction, it is advised to first optimize the initial cell density and second optimize the CHIR99021 concentration. Regarding cell density, we recommend to test the lower (2.4 × 10E6 cells/1 mL master mix) and upper limits (4 × 10E6 cells/1 mL master mix) first. Depending on the outcome, the optimal concentration (3.2 × 10E6 cells/1 mL hydrogel) may be adapted

in one or the other direction in smaller steps. The response to CHIR99021 may vary between lines and may be supplier dependent. A higher concentration of CHIR99021 (optimum 10 μM) results in higher efficiency of skeletal muscle differentiation but may cause excessive cell death. If excessive cell death is observed in 2D monolayer culture the CHIR99021 concentration may be reduced to 7 or 5 μM. Please note however, that in SMO we did not observe excessive cell death even if lines were sensitive in 2D. This is why we would recommend to use 10 μM CHIR99021 as starting point.

- Analyze critical time points of differentiation to ensure proper progression through developmental stages (Figure 2A).

Problem 4

SMO hydrogel sticks to the outer edges of the molds (step 27).

Potential solution

- Ensure homogenous cell distribution and homogeneous casting of the hydrogel.
- If problem remains after day 4 of differentiation, careful mechanical separation of tissue from the edge using a sterile pipette tip or a cannula may be tried. Tissue morphology typically improves further after such a maneuver.
- Use a surfactant to pretreat culture vessels [e.g., 5% solution of Pluronic (F127)]

Problem 5

SMO are ripping during mechanical transfer (step 32).

Potential solution

- Tearing of SMO during mechanical transfer may be caused by inhomogeneous tissue formation with "weak" spots. Try to optimize hydrogel formation (see also problem 2)
- Tearing during handling mostly occurs when moving the stretchers. Slipping while working with forceps or pipette tips may injure and rupture the tissue. Aim to work steadily without uncontrolled movements.
- Use culture platforms that do not require manual handling.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Malte Tiburcy, m.tiburcy@med.uni-goettingen.de.

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Dr. Mina Shahriyari, mina@myriameat.com.

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all datasets generated or analyzed during this study.

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by the NIH Common Fund Regenerative Medicine Program and reported in Stem Cell Reports.[7] The NIH Common Fund and the National Center for Advancing Translational Sciences (NCATS) are joint stewards of the LiPSC-GR1.1 resource. Repairon GmbH acquired and imported a vial of the TC1133 master cell bank, from which a Working Cell Bank (WCB) was created. myriamed GmbH acquired a derivative of the WBC from Repairon GmbH and provided a non-GMP derivative thereof to the Institute of Pharmacology and Toxicology at the University Medical Center Göttingen for non-commercial research use. The graphical abstract was created with BioRender.com.

AUTHOR CONTRIBUTIONS

M.S. established and wrote the protocol. M.R., A.D.H., and A.B. contributed experimental data and edited the manuscript. W.-H.Z. edited the manuscript and provided funding. M.T. conceived the experiments, wrote the manuscript, and provided funding.

DECLARATION OF INTERESTS

M.S., M.T., and W.-H.Z. are co-inventors on a patent on skeletal muscle generation (WO 2021/074126A1). M.S. and M.R. are employees of MyriaMeat GmbH. W.-H.Z. is the founder, shareholder, and advisor of myriamed GmbH, MyriaMeat GmbH, and Repairon GmbH. M.T. is the founder of MyriaMeat GmbH and advisor of myriamed GmbH and Repairon GmbH.

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