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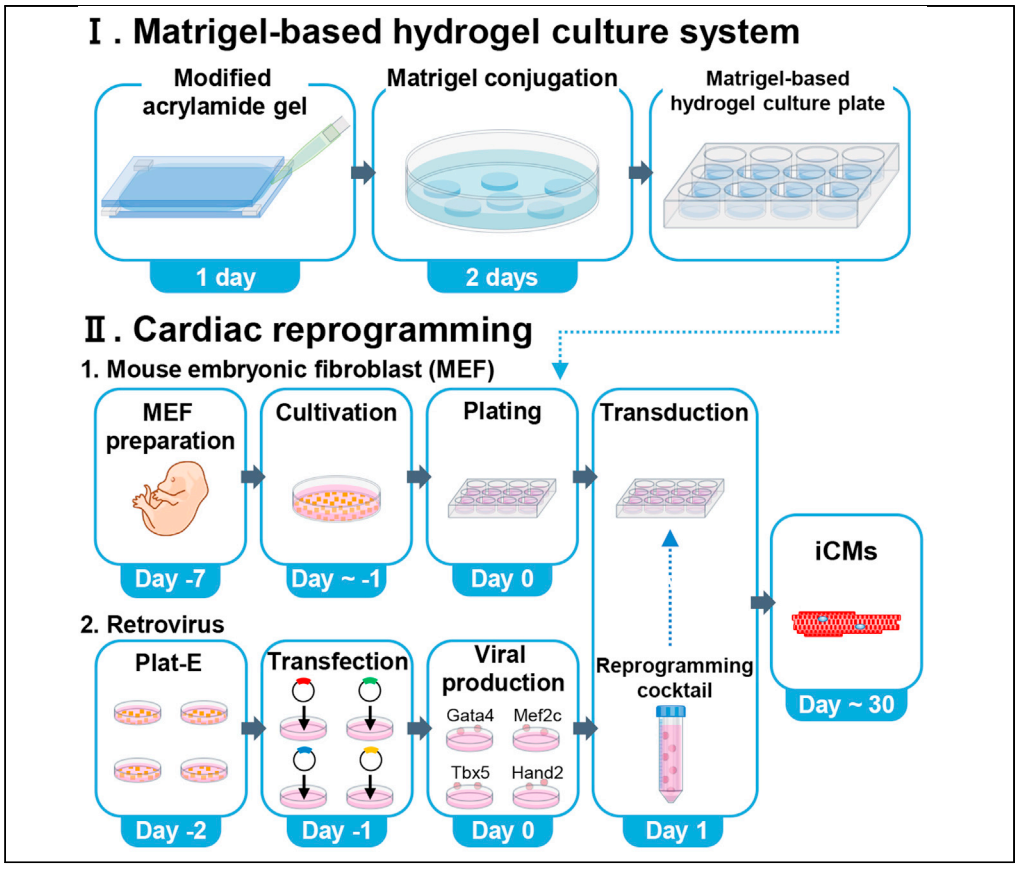
A biomimetic hydrogel culture system to facilitate cardiac reprogramming



Direct cardiac reprogramming, in which fibroblasts are converted into induced cardiomyocytes (iCMs) with cardiogenic transcription factors, may be a promising approach for myocardial regeneration. Here, we present a protocol for cardiac reprogramming using a handmade hydrogel culture system. This system can recapitulate substrate stiffness comparable to that of the native myocardium. This protocol features improved efficiency of cardiac reprogramming by generating threefold more beating iCMs on the Matrigel-based hydrogel culture system compared to that on conventional polystyrene dishes.

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Highlights

Protocol for cardiac reprogramming using a soft hydrogel system

Generation of beating iCMs with 3% efficiency on hydrogel culture

Detailed approaches for generating Matrigel-based hydrogel culture systems

Kurotsu et al., STAR Protocols 3, 101122
March 18, 2022
https://doi.org/10.1016/j.xpro.2022.101122

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A biomimetic hydrogel culture system to facilitate cardiac reprogramming

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https://doi.org/10.1016/j.xpro.2022.101122

SUMMARY

Direct cardiac reprogramming, in which fibroblasts are converted into induced cardiomyocytes (iCMs) with cardiogenic transcription factors, may be a promising approach for myocardial regeneration. Here, we present a protocol for cardiac reprogramming using a handmade hydrogel culture system. This system can recapitulate substrate stiffness comparable to that of the native myocardium. This protocol features improved efficiency of cardiac reprogramming by generating threefold more beating iCMs on the Matrigel-based hydrogel culture system compared to that on conventional polystyrene dishes.

For complete details on the use and execution of this protocol, please refer to Kurotsu et al. (2020)

BEFORE YOU BEGIN

It has been demonstrated that iCMs induced *in vivo* are more mature than those *in vitro*, presumably due to the influence of the surrounding microenvironment (Sadahiro and Ieda, 2021). Cells can sense the stiffness of the underlying matrix, in which two highly related transcriptional coactivators, Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding domain (TAZ), are involved in mechanotransduction (Dupont, 2016; Dupont et al., 2011). The stiffness of biological tissues varies from brain (1 kPa), to heart (10–20 kPa), to collagenous bone (100 kPa), all of which are much softer than conventional polystyrene dishes (~GPa) (Engler et al., 2006, 2008). This protocol focuses on how to create a biomimetic hydrogel culture system that recapitulates the stiffness of the native myocardium, thereby promoting the efficiency of cardiac reprogramming. Since there are multiple papers describing the protocols of cardiac reprogramming on conventional polystyrene dishes, please see these papers for reference (Muraoka et al., 2014; Qian et al., 2013; Yamakawa et al., 2015). The protocol describes specific steps for cardiac reprogramming on a soft Matrigel-based hydrogel culture system; this can promote cardiac reprogramming with retroviral vectors expressing Gata4, Mef2c, Tbx5, and Hand2.

> ***Note:*** Before starting each section of this protocol, prepare the media listed in the "Materials and equipment" and warm to 37°C. Please refer to the "key resources table" for a list of reagents and resources.

Sterilization and cleaning of equipment

Timing: 2 h

1. Immerse the glass slides and silicone spacers in a heat-resistant container filled with water.
2. Autoclave at 121°C for 20 min.
3. Wipe slide glasses with a KimWipe, or a sterilized, non-fluffy cloth, soaked in 70% ethanol.

⚠ CRITICAL: Use slide glasses with no scratches or extraneous matter on the surface.

Preparation of mouse embryonic fibroblasts

Timing: 2 h for MEF isolation and ~2 days for expansion culture

Mouse embryonic fibroblasts (MEFs) are fibroblasts that can be obtained quickly and in large quantities. They can be efficiently reprogrammed into iCMs. The following is a summary of this protocol. For more details, please refer to a previous study (Muraoka et al., 2014).

Note: In this section, we explain the method using 100-mm cell culture dishes. Using this method, 10^7 cells were obtained per culture dish.

Note: The following steps should be performed in a safety cabinet under sterile conditions.

4. Wash mouse embryos (12.5–13.5 days post coitum) in a 100-mm dish containing 10 mL sterile PBS.
5. Transfer embryos to a clean dish without PBS. Carefully remove the head, heart, and the other visceral tissues using a stereomicroscope.
6. Gather 3–5 carcasses and mince them with scissors.
7. Transfer the minced embryos to 15 mL of pre-warmed 0.25% trypsin/EDTA solution.
8. Incubate at 37°C for 20 min.
9. Add 15 mL of FBS and pipette several times to allow for tissue dissociation.
10. Centrifuge the dissociated tissue at 1,000*g* for 3 min.
11. Discard supernatants and resuspend in 10 mL MEF medium.
12. Plate the cells on a 100-mm cell culture dish.
13. Incubate at 37°C in 5% CO_2.

⚠ CRITICAL: Replace media every other day after the first day. Cells should become confluent after 1–2 days. Do not continue culturing for more than 7 days, as the reprogramming efficiency decreases after this time.

Pause point: After confluence, MEFs can be stored in MEF freezing medium (Combine FBS with 10% DMSO) at 5 × 10^6 cells/mL in liquid nitrogen. The use of frozen MEF stock is convenient. We have confirmed that the hydrogel-based culture system promotes reprogramming efficiency to generate more than a three-fold increase in beating iCMs than the polystyrene dish, even with frozen MEF stocks, but the average reprogramming efficiency is lower than when using fresh MEFs.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti- Troponin T (dilution 1:250)	Thermo Fisher Scientific	Cat# MS-295-P1, RRID:AB_61808
Alexa Fluor 647 goat anti-mouse IgG (dilution 1:250)	Thermo Fisher Scientific	Cat# A21240; RRID: AB_2535809
Alexa Fluor 546 goat anti-mouse IgG (dilution 1:250)	Thermo Fisher Scientific	Cat# A11003; RRID: AB_141370

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
DAPI (dilution 1:250)	Thermo Fisher Scientific	Cat# D1306, RRID: AB_2629482
Chemicals, peptides, and recombinant proteins		
0.25w/v% Trypsin-EDTA	Gibco	Cat# 25200-072
0.5w/v% Trypsin-EDTA	Fujifilm Wako	Cat# 208-17251
Gelatin	Sigma-Aldrich	Cat# G1393
Opti-MEM	Gibco	Cat# 31985-070
Fugene6	Promega	Cat# E2691
Acrylamide	Nacalai Tesque	Cat# 00809-85
N,N′-methylenebisacrylamide	Nacalai Tesque	Cat# 22402-02
6-acrylamidohexanoic acid	Tokyo Chemical Industry	Cat# A1896
Ammonium peroxodisulfate	Nacalai Tesque	Cat# 02627-21
N,N,N′,N′-tetramethylethylenediamine	Nacalai Tesque	Cat# 33401-72
2-(N-morpholino) ethanesulfonic acid	Nacalai Tesque	Cat# 21623-26
Matrigel	Corning	Cat# 356230
Sodium chloride	Fujifilm Wako	Cat# 191-01665
Sodium hydroxide	Fujifilm Wako	Cat# 194-18865
Methanol	Fujifilm Wako	Cat# 131-01826
10x PBS powder	Nissui	Cat# 08192
DPBS	Gibco	Cat# 14190-144
D-MEM (High Glucose) with L-Glutamine and Phenol Red	Fujifilm Wako	Cat# 044-29765
Sodium pyruvate	Sigma-Aldrich	Cat# S-8636
Non-essential amino acids solution (NEAA)	Sigma-Aldrich	Cat# M-7145
FBS	COSMO BIO	Cat# CCP-FBS-BR-500
Medium 199 (M199)	Gibco	Cat# 11150-059
Ascorbic acid	Sigma-Aldrich	Cat# A-4544
Recombinant human FGF basic 146 aa	R&D Systems	Cat# 233-FB-025
Recombinant human FGF10	R&D Systems	Cat# 345-FG-025
Recombinant human VEGF165	R&D Systems	Cat# 293-VE-050
N-hydroxysuccinimide	Nacalai Tesque	Cat# 18948-44
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride	Fujifilm Wako	Cat# 346-03632
StemPro-34 SF medium	Gibco	Cat# 10639-011
GlutaMAX	Gibco	Cat# 35050-061
Antibiotic-Antimycotic (PSA)	Gibco	Cat# 15240096
Puromycin	Sigma-Aldrich	Cat# P9620
Blasticidin	Gibco	Cat# A1113903
Bovine serum albumin	Fujifilm Wako	Cat# 034-25462
DMSO	Sigma-Aldrich	Cat# 20-139
Experimental models: Cell lines		
Plat-E cell line	Cell Biolabs	Cat# RV-101
Experimental models: Organisms/strains		
Mouse: Jcl:ICR or C57BL/6JJcl	CLEA Japan	N/A
Recombinant DNA		
pMX-Gata4	Ieda et al. (2010)	N/A
pMX-Mef2c	Ieda et al. (2010)	N/A
pMX-Tbx5	Ieda et al. (2010)	N/A
pMX-Hand2	Ieda et al. (2010)	N/A
pMX-GFP	Ieda et al. (2010)	N/A
Other		
100 mm dish	Thermo Fisher Scientific	Cat# 150466
100 μm cell strainer	Falcon	Cat# 352360
0.45-mm-pore filter	Sartorius	Cat# 17598K
Slide glass	Matsunami Glass	Cat# S092240
Silicone membrane	Tigers Polymer Association	Cat# SR-50
Kimwipe	Nippon Paper Crecia	Cat# 62011
12-well plate	CORNING	Cat# 353043

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
5 mL syringe	Terumo	Cat# SS-5LZ
20 mL syringe	Terumo	Cat# SS-20LZ
0.22-μm filter	IWAKI (AGC)	Cat# 11-0379

Materials and equipment

- Gelatin (0.1%): Add 0.5 g gelatin to a total volume of 500 mL PBS and filter the autoclaved solution with a 0.22-μm filter. Store at 4°C and use within 1 month.
- Mouse embryonic fibroblast (MEF) medium: Combine Dulbecco's modified Eagle medium (DMEM) with 10% FBS, 1% sodium pyruvate, 1% non-essential amino acid (NEAA), and 1% Antibiotic-Antimycotic (PSA). Store at 4°C and use within 1 month.

Reagent	Final concentration	Amount
DMEM	N/A	435 mL
FBS	10%	50 mL
Sodium pyruvate	1%	5 mL
NEAA	1%	5 mL
PSA	1%	5 mL
Total	**n/a**	**500 mL**

- Plat-E transfection medium: Combine DMEM supplemented with 10% FBS and 1% PSA. Store at 4°C and use within 1 month.

Reagent	Final concentration	Amount
DMEM	N/A	445 mL
FBS	10%	50 mL
PSA	1%	5 mL
Total	**n/a**	500 mL

- Plat-E maintenance medium: Add 1 μg/mL puromycin and 10 μg/mL blasticidin to Plat-E transfection medium. Store at 4°C and use within 1 month.

Reagent	Final concentration	Amount
Plat-E transfection medium	N/A	499.45 mL
Puromycin (10 mg/mL)	1 μg/mL	50 μL
Blasticidin (10 mg/mL)	10 μg/mL	500 μL
Total	**n/a**	500 mL

- iCM medium: Combine DMEM supplemented with 10% FBS, 20% M199, 1% NEAA, 1% sodium pyruvate, 1% GlutaMAX, and 1% PSA. Store at 4°C and use within 1 month.

Reagent	Final concentration	Amount
DMEM	N/A	330 mL
FBS	10%	50 mL

(*Continued on next page*)

Continued

Reagent	Final concentration	Amount
M199	20%	100 mL
NEAA	1%	5 mL
Sodium pyruvate	1%	5 mL
GlutaMax	1%	5 mL
PSA	1%	5 mL
Total	**n/a**	**500 mL**

- FFV medium for long-term iCM culture: Supplement StemPro-34 SF medium with 5 ng/mL recombinant human VEGF165, 10 ng/mL recombinant human FGF basic 146 aa, 25 ng/mL recombinant human FGF10, 100 μg/mL ascorbic acid, and 1% GlutaMAX. Store at 4°C and use within one week.

Reagent	Final concentration	Amount
StemPro-34 SF medium	N/A	9.675 mL
recombinant human VEGF165 (5 ng/μL)	5 ng/mL	10 μL
recombinant human FGF basic 146 aa (10 ng/μL)	10 ng/mL	10 uL
recombinant human FGF10 (50 ng/μL)	25 ng/mL	5 uL
Ascorbic acid (5 mg/mL)	0.1 mg/mL	200 μL
GlutaMax	1%	100 μL
Total	**n/a**	**10 mL**

Note: For stock solutions and aliquots preparation, recombinant human VEGF165, recombinant human FGF basic 146 aa, and recombinant human FGF10 are reconstituted in sterile PBS containing 0.1% bovine serum albumin (BSA). Ascorbic acid is reconstituted in ddH_2O. Make aliquots and store at −80°C and use within 6 months. Use each aliquot once and discard the rest.

- 40% acrylamide (AA) solution

Reagent	Final concentration	Amount
AA	40%	40 g
ddH_2O	n/a	100 mL
Total	**n/a**	**100 mL**
Store at 4°C in the dark and use within 3 months		

- 2% N,N′-methylenebisacrylamide (Bis-acrylamide; BIS) solution

Reagent	Final concentration	Amount
BIS	2%	2 g
ddH_2O	n/a	100 mL
Total	**n/a**	**100 mL**
Store at 4°C in the dark and use within 3 months		

- 500 mM 6-acrylamidohexanoic acid (ACA) solution

Reagent	Final concentration	Amount
ACA	500 mM	0.925 g
ddH_2O	n/a	6.5 mL
1 N NaOH	n/a	3.5 mL
Total	**n/a**	**10 mL**
Store at 4°C in the dark and use within 3 months		

- 10% Ammonium peroxodisulfate solution (APS)

Reagent	Final concentration	Amount
APS	10%	1 g
$2ddH_2O$	n/a	10 mL
Total	**n/a**	**10 mL**
Dispense 10 μL each and store at −20°C and use within 3 months		

- 2-(N-morpholino) ethanesulfonic acid (MES) buffer

Reagent	Final concentration	Amount
MES	0.1 M	21.325 g
NaCl	0.5 M	29.220 g
ddH_2O	n/a	500 mL
5 N NaOH	n/a	adjust to pH 6.1
ddH_2O	n/a	up to 1000 mL
Total	**n/a**	**1000 mL**
Store at room temperature (24°C–28°C) and use within 3 months		

- 60% Met-OH/PBS

Reagent	Final concentration	Amount
Methanol (Met-OH)	60%	60 mL
5 times concentrated PBS	n/a	20 mL
ddH_2O	n/a	up to 100 mL
Total	**n/a**	**100 mL**
Store at 4°C and use within 3 months		

⚠ CRITICAL: Acrylamide is toxic. BIS and APS are hazardous. ACA is an irritant. 5 N NaOH is corrosive. Methanol is both harmful and flammable. Working with these materials requires the use of personal protective equipment such as impermeable gloves (nitrile, latex, etc.) and a lab coat. Avoid direct contact with these materials, including inhalation of fumes. Do not work in a poorly ventilated space.

STEP-BY-STEP METHOD DETAILS

Polymerization of the ACA-modified acrylamide gel

Timing: 3 h (Polymerizing the gel: 1 h, Peeling and washing the gel: 2 h)

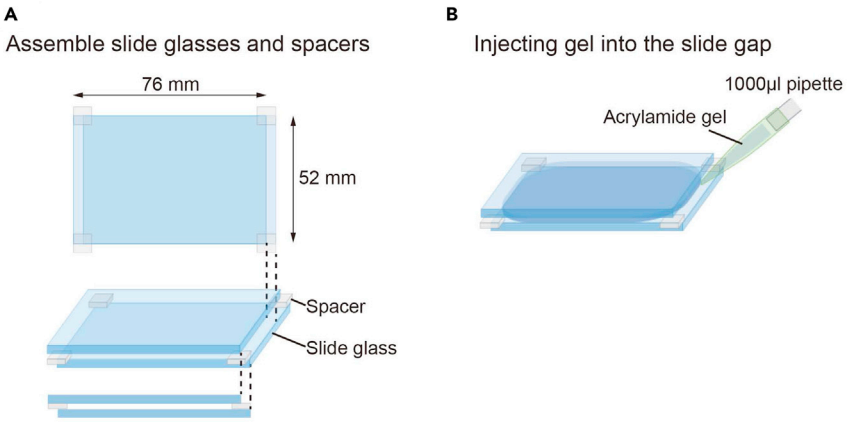


Figure 1. Polymerization of the ACA-modified acrylamide gel

(A and B) Assemble slide glasses and spacers as shown in the illustration (A). Gently pour the polymerizing solution (1 mL) into the gap of slide glasses (B).

The stiffness of the myocardium is approximately 10 kPa. In this step, we explain the preparation of polyacrylamide gels that resemble myocardial stiffness.

Note: The following steps should be performed in a safety cabinet under sterile conditions. In addition, replace gloves before 2handling the gel with your fingers.

1. Assemble slide glasses and silicone membranes as spacers as shown in Figure 1A.
2. To later allow conjugation with Matrigel components, mix the ACA into a polyacrylamide gel. Mixed reagents are shown in Table 1. Be careful not to generate air bubbles.
3. Gently pour 1 mL polymerizing solution into the gap of the glass slide, as shown in Figure 1B. Be careful not to generate air bubbles.

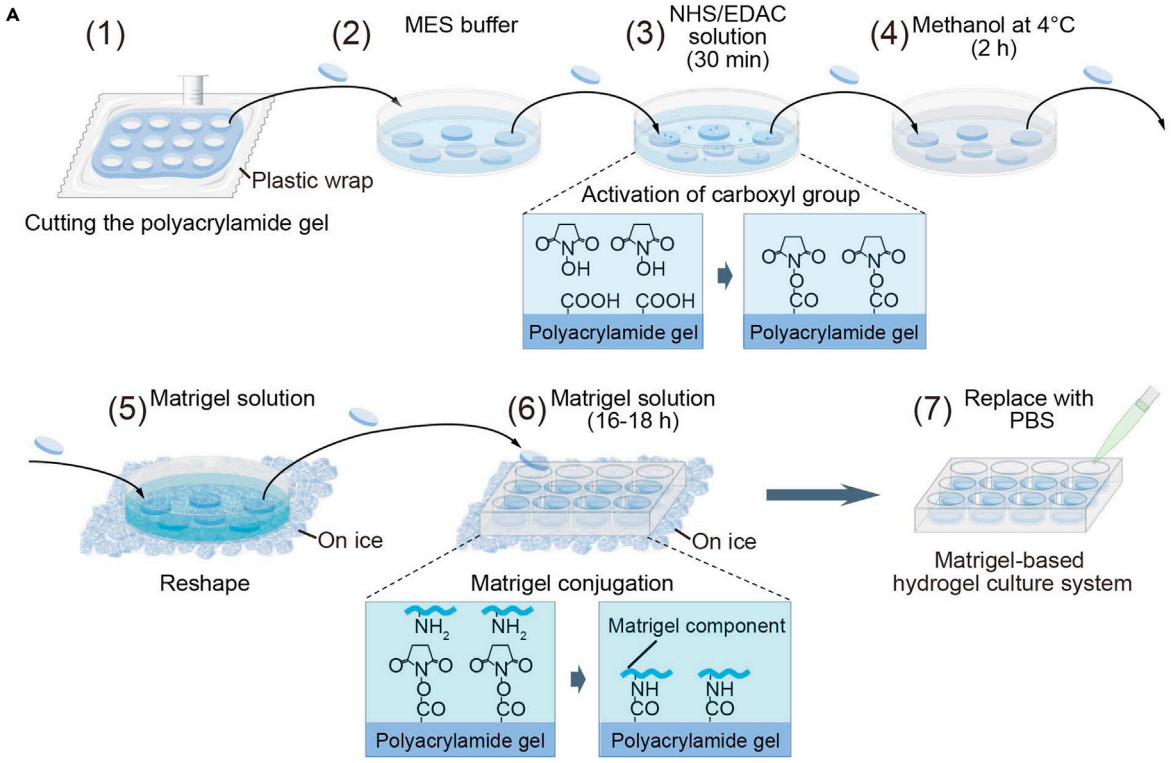
⚠ CRITICAL: Polymerization starts immediately after the addition of TEMED and APS. Therefore, TEMED and APS should be added separately after preparing the other mixtures. TEMED is flammable, corrosive, and hazardous; wear impermeable gloves and a lab coat, avoid direct contact and inhalation of fumes, and do not work in a poorly ventilated space.

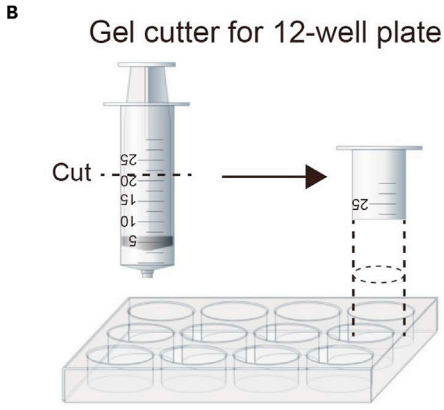
Note: The healthy myocardium has a stiffness of approximately 10 kPa. The stiffness of the gel made with the reagents in Table 1 is about 7.5 kPa, which is equivalent to that of healthy myocardium (Kurotsu et al., 2020; Yip et al., 2013). The stiffness of the gel can be varied by adjusting the composition of BIS. For details on the hydrogel composition of the other stiffness, please refer to Kurotsu et al. (2020).

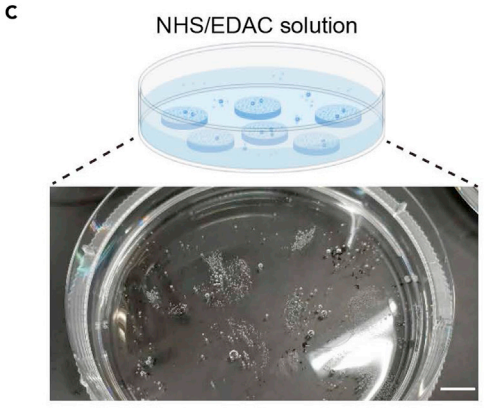
4. Allow the solution to polymerize completely at room temperature.

Table 1. Mixture for polyacrylamide gel

Reagent	Final concentration	Amount
ddH_2O (µL)	n/a	405
40% AA (µL)	7.5%	188
2% BIS (µL)	0.04%	20
500 mM ACA (µL)	190 mM	380
N,N,N′,N′-tetramethylethylenediamine (TEMED) (µL)	0.2%	2
10% APS (µL)	0.05%	5
Total (µL)	**n/a**	**1000**


A
(1)
(2) MES buffer
(3) NHS/EDAC solution (30 min)
(4) Methanol at 4°C (2 h)
Plastic wrap
Cutting the polyacrylamide gel
Activation of carboxyl group
Polyacrylamide gel
Polyacrylamide gel
(5) Matrigel solution
(6) Matrigel solution (16-18 h)
(7) Replace with PBS
On ice
On ice
Reshape
Matrigel conjugation
Matrigel component
Polyacrylamide gel
Polyacrylamide gel
Matrigel-based hydrogel culture system


B
Gel cutter for 12-well plate
Cut


C
NHS/EDAC solution

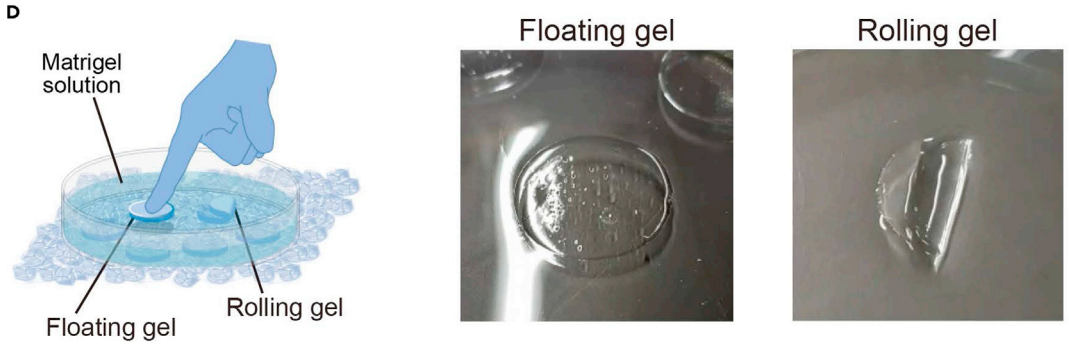

D
Matrigel solution
Floating gel
Rolling gel
Floating gel
Rolling gel

Figure 2. Generation of 12-well culture plates containing Matrigel-based hydrogels

(A) Workflow of generating a Matrigel-based hydrogel culture system from (1) to (7). The incubation time is shown for each step.

(B) Process the outer cylinder of a syringe to make a "gel cutter". Because hydrogel still swells slightly after Matrigel conjugation, it is better to use a syringe with a diameter slightly smaller than the bottom of the well. See also Methods video S3.

(C) ACA (co-polymerized in the hydrogel) reacts with NHS/EDAC to form active ester bonds. If the reaction proceeds successfully, bubbles are generated. These bubbles interfere with further reaction between the hydrogel and NHS/EDAC, which should be removed by shaking the dish every 15 min. See also Methods video S4.

(D) Active ester forms on the surface of the hydrogel and reacts with Matrigel components. In this process, the gel repels water and may float. Tap the gel to submerge it in the solution. Continue this process until the gel becomes familiar with the solution and does not float. Some gels may curl, but this can be reversed by performing the same operation. If the gels curl or overlap, separate them as soon as possible. See also Methods videos S5 and S6. Scale bar represents 1 cm.

⚠ CRITICAL: When making soft gels at a low room temperature, polymerization takes longer. The approximate time required for polymerization is 30 min at 25°C to generate a 7.5 kPa gel.

5. After polymerization, remove the slide glass. See Methods video S1.
6. Peel off the gel from the glass slide. See Methods video S2. Troubleshooting 1

⚠ CRITICAL: Since the hydrogel (polyacrylamide gel) is easy to tear, soak it in MES buffer, and gently peel it off from the perimeter.

Note: For clean operation, change gloves at this step.

7. Soak the gel in a 10 mL MES buffer in a 100-mm cell culture dish for 30 min. Change the buffer solution three times.
8. Soak the gel in a fresh 10 mL MES buffer and incubate overnight (12–16 h) in a refrigerator at 4°C to hydrate completely.

Preparation of culture plates with Matrigel-based hydrogels for cardiac reprogramming

Timing: 2 days (Cutting the gel: 1 h, Activating carboxyl group: 2.5 h, Transferring the gels: variable (typically 20–30 min per 12-well plate), PBS replacement: variable (approximately 40 min incubation plus 10–15 min per 12-well plate)

Since cells do not adhere to polyacrylamide gel, it is necessary to modify the polyacrylamide gel to be coated with an extracellular matrix (ECM), such as Matrigel (Figure 2A). In addition, a strong bond between the ECM and polyacrylamide gel is necessary because ECM detaches from the gel after long-term culture. This step covalently bonds the Matrigel to the modified polyacrylamide gel so that the ECM does not peel off. Plates with Matrigel-based hydrogels can be stored for more than four weeks.

Note: In this step, Matrigel components covalently bind to a modified polyacrylamide gel in a 12-well plate.

Note: The following steps should be performed in a safety cabinet under sterile conditions. In addition, replace gloves before handling the gel with your fingers.

9. Mix the reagents as shown in Table 2.

Prepare at the time of use

⚠ CRITICAL: NHS and EDAC are hazardous. Avoid contact and inhalation. Wear impermeable gloves and a lab coat.

Table 2. 0.5 M N-Hydroxysuccinimide (NHS)/0.2 M 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride (EDAC) solution

Reagent	Final concentration	Amount
NHS	0.5 M	5.75 g
EDAC	0.2 M	3.83 g
MES buffer	n/a	up to 100 mL
Total	**n/a**	**100 mL**

10. On a sheet of plastic wrap, cut the hydrogel to the same size as the well of a 12-well plate. Immerse the cut-out round gel in the MES buffer immediately to prevent the hydrogel from drying out. See Methods video S3 and Figure 2A-(1) and (2).

Note: The hydrophilic gel expands beyond its original size. 8–10 wells of a 12-well plate can be cut out from a 7.5 kPa gel.

Note: The hydrogels further swell slightly after Matrigel conjugation; therefore, it is best to cut the gel slightly smaller than the bottom of the cell culture plate (Figure 2B). Because the diameter of the outer cylinder of a syringe matches the diameter of the well of a 12-well plate, the outer cylinder can be processed to be used as a "gel cutter." The 20 mL and 5 mL syringes are suitable for a 12-well and 24-well plate gel cutters, respectively. Soak gel cutters in 70% ethanol after use, and store.

Note: For clean operation, change gloves at each subsequent step.

11. Transfer the cut-out hydrogels to a 100-mm cell culture dish containing 10 mL of 0.5 M NHS/0.2 M EDAC solution. See Figure 2A-(3).
12. Incubate samples for 30 min at room temperature to activate the carboxyl group in the polyacrylamide gel. The reaction between the gel and the solution produces gas. Shake the container to remove bubbles on the surface of the hydrogel. See Figure 2C and Methods video S4.

⚠ CRITICAL: Bubbles interfere with the chemical reaction between the hydrogel and the NHS/EDAC solution and should be removed every 15 min.

13. Transfer the hydrogel to a 100-mm cell culture dish containing 10 mL of 60% methanol/PBS pre-cooled at 4°C. See Figure 2A-(4).
14. Incubate for 2 h in a refrigerator at 4°C.

Note: After incubation, the gel shrinks a little due to dehydration.

15. Transfer the hydrogel to a 100-mm cell culture dish on ice containing 10 mL of 0.05 mg/mL Matrigel/PBS solution. See Figure 2A-(5).

⚠ CRITICAL: This operation should be performed on ice to prevent the hydrogels from adhering to each other due to accelerated Matrigel conjugation.

⚠ CRITICAL: At this time, the gel repels water and floats. Tap the gel to submerge it in the solution. Continue this process until the gel remains submerged in the solution. Some gels may continue to float and roll, but this can be addressed by gently fixing the gels using a finger (Figure 2D). See Methods Videos S5 and S6.

Note: Alternatively, the gel can be handled with clean forceps for aseptic manipulation in this step. However, if manipulation with these instruments would damage the gel, it is recommended to change into clean gloves and work with your fingers.

16. After transferring the gels from the 100-mm culture dish to each well, add 500 μL of 0.05 mg/mL Matrigel/PBS to each well of the 12-well plate on ice. See Figure 2A-(6).
17. Incubate for 16–18 h in the refrigerator set at 4°C for Matrigel conjugation.
18. Replace one-third of the Matrigel/PBS solution with PBS and incubate for 10 min in the refrigerator set at 4°C. Repeat this process three times. See Figure 2A-(7).
19. Completely replace the Matrigel/PBS solution with PBS and incubate overnight (12–16 h) in the refrigerator set at 4°C.

Note: Make sure that the hydrogel adheres to the cell culture plate. If the hydrogel floats up, it is difficult to reattach it to the culture plate.

Pause point: This culture plate can be stored in the refrigerator set at 4°C for up to 1 month. We recommend starting the subsequent cardiac reprogramming process after preparing multiple culture plates.

Cardiac reprogramming

Timing: 7–30 days

For cardiac reprogramming from fibroblasts, both retroviral vector generation and fibroblast preparation should be parallel.

⚠ CRITICAL: The following steps should be performed in a safety cabinet under sterile conditions.

Preparation for retrovirus infection

Timing: 4 days (1 h on day 0, 1 h on day 1, 30 min on day 2, and 1 h on day 3)

To construct retroviral vectors, we used pMXs retroviral vectors for GFP, Gata4, Mef2c, Tbx5, and Hand2. The following is a summary of this protocol. For more details, please refer to previous studies (Ieda et al., 2010; Muraoka et al., 2019; Qian et al., 2013).

20. On day 0 for cardiac reprogramming, coat five 100-mm cell culture dishes with 0.1% gelatin/PBS.
21. Plate Plat-E cells at a density of 3.6×10^6 cells/dish in Plat-E transfection medium.

Note: We recommend that Plat-E cells culture should be limited to 30 passages.

⚠ CRITICAL: Do not use the Plat-E maintenance medium.

22. Incubate at 37°C in 5% CO_2 for 24 h.
23. On day 1 for cardiac reprogramming, add 300 μL of OptiMEM to five 1.5 mL Eppendorf tubes, mixed with 27 μL of Fugene6 in each tube, and tap well with fingers. Prepare five tubes in total.
24. Incubate at room temperature for 5 min.
25. Mix 9 μg of each retroviral DNA into this mixture by finger tapping and incubate at room temperature for 15 min. Add the transfection mixture containing each retroviral DNA and Fugene6 to the culture medium and mix well by shaking the culture dish.
26. Incubate at 37°C in 5% CO_2 for 24 h.

27. On day 2 for cardiac reprogramming, replace the medium containing the transfection mix with 10 mL of fresh Plat-E transfection medium.
28. Incubate at 37°C in 5% CO_2 for 24 h.
29. On day 3 for cardiac reprogramming, collect each retroviral medium and filter through a 0.45-mm pore filter using a 10 mL sterile disposable syringe.
30. Mix 4 μL of polybrene with each retroviral medium to a final concentration of 4 μg/mL.
31. Prepare a cardiac reprogramming cocktail by mixing equal amounts of the retroviral medium of Gata4, Hand2, Mef2c, and Tbx5 (GHMT). The retroviral mixture is now ready for use.

Note: GFP retrovirus vectors can be used to monitor transduction efficiency. A transduction efficiency of 95%–100% is required for successful reprogramming.

Culture of MEFs on Matrigel-based hydrogels and retrovirus infection

Timing: 2 h on day 2, 1 h on day 3, 1h on day 4 and 30 min every 2–3 days

⚠ CRITICAL: Be careful not to aspirate the hydrogel when changing the medium. We use a 200 μL or 1,000 μL pipette to slowly change the medium.

32. On day 2 for cardiac reprogramming (the same day as step 27), remove PBS from the Matrigel-based hydrogel plate.

⚠ CRITICAL: Tilt the plate to completely remove PBS between the plate and hydrogel (Figure 3A).

33. Expose to UV light in a sterile hood for 30 min. We used a UV lamp in a safety cabinet.

⚠ CRITICAL: The polystyrene lid of the plate absorbs UV; thus, remove the lid during UV exposure (Methods video S7).

34. Gently pour 1 mL of MEF medium into the hydrogel-attached wells. Incubate the mixture for 30 min at room temperature (Figure 3B). Troubleshooting 2
35. Remove the MEF medium.
36. Seed MEFs at a density of 35,000 cells in 1 mL of MEF medium on Matrigel-based hydrogels for 12-well plate.

⚠ CRITICAL: Filter the cultured MEFs through a 100-μm cell strainer to remove debris.

37. Incubate at 37°C in 5% CO_2 for 24 h. Troubleshooting 3 and 4
38. On day 3 for cardiac reprogramming (the same day as step 29–31), the medium was replaced with 1 mL of a reprogramming cocktail prepared in step 31.
39. Incubate at 37°C in 5% CO_2 for 24 h.
40. On day 4 for cardiac reprogramming, replace the reprogramming cocktail with 1 mL of iCM medium. After 14 days of infection, replace the medium with FFV medium for iCM maturation.
41. Incubate at 37°C in 5% CO_2 for one month to generate beating iCMs. Assess the efficiency of cardiac reprogramming after 1 week using FACS. Troubleshooting 5

Note: Change the iCM medium every 2–3 d. Replace FFV medium twice a week.

EXPECTED OUTCOMES

Assess cardiac reprogramming 1 week after transduction. Analyze reprogramming efficiency by FACS and immunostaining for iCMs. For evaluation by FACS, analyze iCMs expressing cardiac

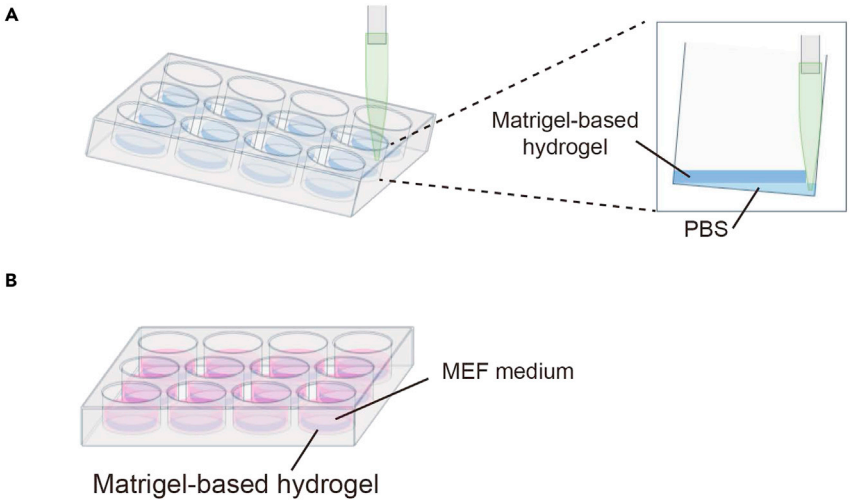


Figure 3. Culture of MEFs on Matrigel-based hydrogels

(A) Insert a tip into the gap between the well and the hydrogel and completely remove PBS with micropipetting. If PBS remains in the gap between the well and the hydrogel, the hydrogel cannot adhere to the bottom.

(B) Gently pour 1 mL of MEF medium into the hydrogel-attached wells.

troponin T (cTnT) 1 week after transduction (Figure 4A). It takes approximately 4 weeks to induce iCMs exhibiting a clear sarcomeric structure, as shown by immunostaining for cTnT (Figure 4B). Spontaneous beating iCMs, an indicator of functionally mature CMs, can be observed approximately 4 weeks after transduction and increase over time. If cardiac reprogramming is successful with this system, the efficiency of $cTnT^+$ cell induction will be more than 10% after 1 week as assessed by FACS, and the efficiency of beating iCMs will be ~3% among total cells after 4 weeks (Methods video S8). Note that the increase in cTnT expression and beating iCMs is increased by ~1.5- and ~3-fold, respectively, in a hydrogel system compared to using a conventional polystyrene dish (Figures 4C-4E). A list of recommended antibodies for these assays can be found in the key resource table. For more details, please refer to previous studies (Qian et al., 2013).

LIMITATIONS

Although the use of the hydrogel culture system improves the efficiency of cardiac reprogramming, it has some limitations.

First, only MEFs were used in this study. The reprogramming efficiency in other fibroblasts, such as cardiac fibroblasts, tail-tip fibroblasts, and human fibroblasts, requires further investigation.

Second, to mimic the *in vivo* environment entirely, it is necessary to reproduce beating heart muscles, electrical stimulation, exogenous hormones, growth factors, etc., which are not included in this system.

Third, other ECMs (collagen, laminin, gelatin, etc.) can be used instead of Matrigel; however, we have not thoroughly investigated the effects of other ECMs.

Finally, we did not investigate the effect of ECM stiffness on direct reprogramming in other cell types, including blood cells, endothelial cells, and stem cells.

TROUBLESHOOTING

Problem 1

The polyacrylamide gel does not easily peel off from the slide glass (step 6).

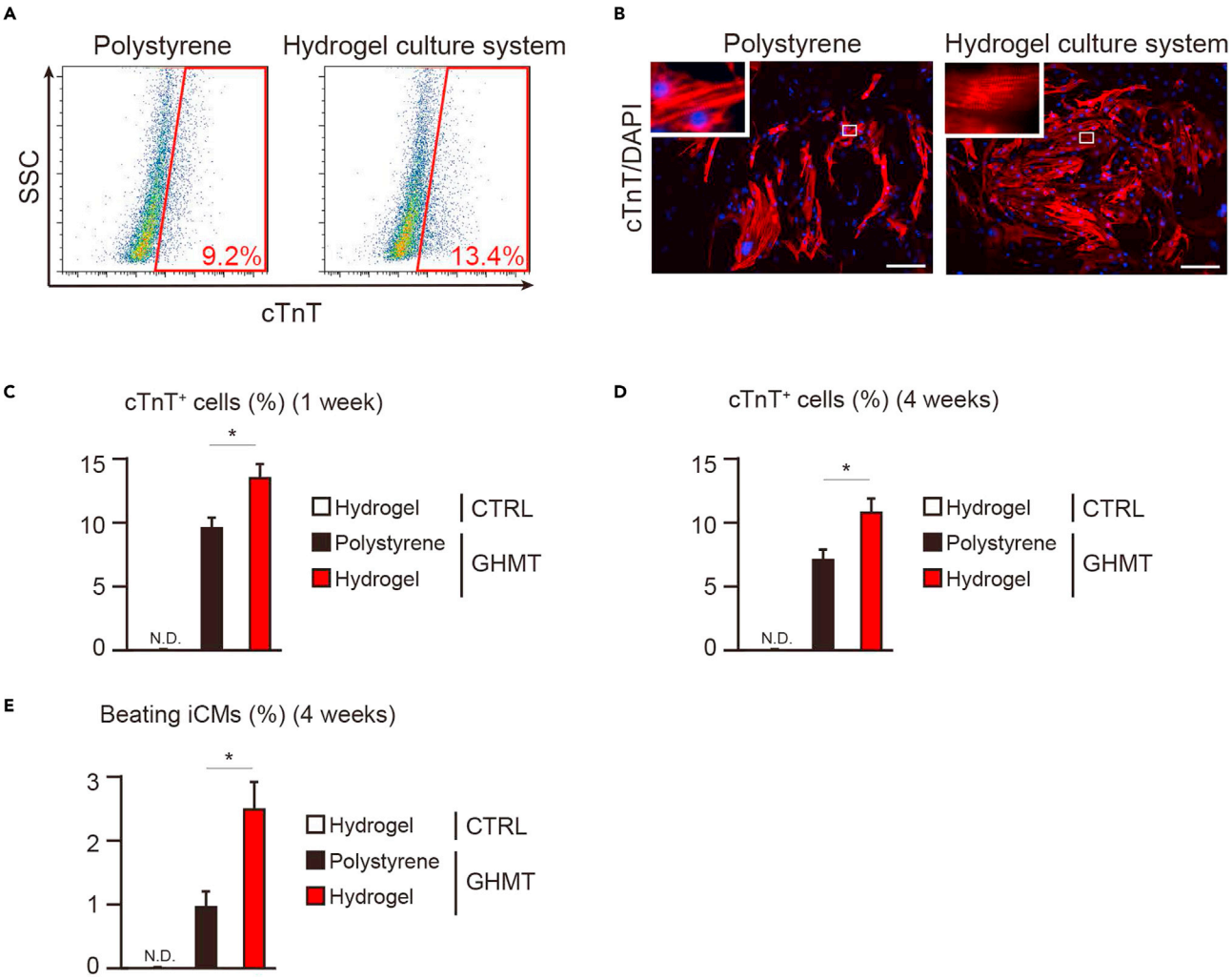


Figure 4. Cardiac reprogramming and expected outcomes

(A) FACS analysis of cTnT expression after 1 week on the polystyrene and hydrogel dishes.

(B) Immunocytochemistry of cTnT and DAPI after 4 weeks on the polystyrene and hydrogel dishes. High-magnification views, as insets, show sarcomeric organization.

(C) FACS analysis of cTnT expression after 1 week. Fibroblasts were transduced with GHMT and cultured on polystyrene and hydrogel dishes. Quantitative data; n = 3 independent triplicate experiments.

(D) Immunocytochemistry of cTnT and DAPI after 4 weeks. Cells were treated as described in (C). Quantitative data; n = 3 independent triplicate experiments.

(E) Quantitative data for the number of spontaneously beating iCMs after 4 weeks; n = 3 independent triplicate experiments.

All data are presented as the mean ± SD. *p < 0.05 versus the relevant control. ND, not detected; CTRL, control. Scale bars represent 100 μm.

(C–E) These figures were modified and cited from Kurotsu et al. (2020).

Potential solution

After removing one side of the slide glasses, soak the gel and the slide glass in the MES buffer while they are still attached, and wait for a while. When the perimeter of the gel gets wrinkled, gently peel the gel completely from the slide glass with a finger. Wear clean gloves and wet fingertips with a MES buffer before touching the gel. It is desirable to handle the gel with clean forceps or tweezers to maintain sterility, but it is difficult to handle the gel without damaging it, so it is recommended to handle the gel with a finger with clean gloves.

Problem 2

The hydrogel does not attach to the culture plate (step 34).

Potential solution

Check the reaction of the NHS/EDAC solution with polyacrylamide gel (steps 11 and 12 and Figure 2A-(3)). If no bubbles were generated from the hydrogel, repeat the reaction. Bubbles may interfere with the reaction, thus shake the culture dish to remove them from the surface of the hydrogel during the reaction.

Excess Matrigel components may prevent the hydrogel from adhering to the well (Figure 2A-(7)). Wash the hydrogel thoroughly with PBS. Be careful not to damage the surface of the hydrogel during washing (steps 18 and 19).

If PBS remained in the gap between the well and the hydrogel, the hydrogel did not adhere to the well (step 32 and Figure 3A). Use a suitable gel cutter to completely remove PBS (Figure 2B).

Problem 3

MEFs do not adhere to the hydrogel (step 37).

Potential solution

See step 15 and Figures 2A-(5)(6) and 2D.

Soak the floating gel and allow the Matrigel/PBS solution to blend with the gel.

If the gel is curled up, the cells cannot adhere to it. Tap the gel to reshape it.

Problem 4

MEFs are contaminated after spreading on Matrigel plates (step 37).

Potential solution

There is a high possibility of gel contamination. Discard gels generated in the same experiment. To prevent contamination, use sterile PBS during the gel fabrication process and UV sterilization before use.

Problem 5

Cells attached to the gel are not easily dissociated for analysis (step 41).

Potential solution

Before trypsinization, wash the wells with warm PBS for 10 min. Repeat this procedure three times. If there is still no improvement, incubate with 0.5% w/v trypsin-EDTA for 15 min at 37°C.

RESOURCE AVAILABILITY

Lead contact

Further information and inquiries should be directed to and will be fulfilled by the lead contact, Masaki Ieda (mieda@md.tsukuba.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101122.

ACKNOWLEDGMENTS

This study was supported by research grants from JSPS (21K08072), University of Tsukuba Basic Research Support Program Type S, and Japan Foundation for Applied Enzymology to T.S.; Research Center Network for Realization of Regenerative Medicine (JP20bm0704030), the Japan Agency for Medical Research and Development, the Japan Society for the Promotion of Science (19K22613, 19K17550), and Takeda Science Foundation to M.I.

AUTHOR CONTRIBUTIONS

S.K., T.S., and M.I. designed the experiments. S.K., T.S., I.H., and M.I. analyzed the data. S.K., T.S., I.H., and M.I. wrote the manuscript.

DECLARATION OF INTERESTS

S.K. is an employee of Otsuka Pharmaceutical Co., Ltd. I.H. is an employee of Canon Inc. M.I. holds a patent related to this work: U.S. Patent 9,517,250 entitled "Methods for Generating Cardiomyocytes," issued on October 19, 2012. Inventors: Deepak Srivastava and Masaki Ieda.

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