Spatiotemporal Extracellular Matrix Modeling for in Situ Cell Niche Studies

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Abstract
Extracellular matrix (ECM) components govern a range of cell functions, such as migration, proliferation, maintenance of stemness, and differentiation. Cell niches that harbor stem-/progenitor cells, with matching ECM, have been shown in a range of organs, although their presence in the heart is still under debate. Determining niches depends on a range of in vitro and in vivo models and techniques, where animal models are powerful tools for studying cell-ECM dynamics; however, they are costly and time-consuming to use. In vitro models based on recombinant ECM proteins lack the complexity of the in vivo ECM. To address these issues, we present the spatiotemporal extracellular matrix model for studies of cell-ECM dynamics, such as cell niches. This model combines gentle decellularization and sectioning of cardiac tissue, allowing retention of a complex ECM, with recellularization and subsequent image processing using image stitching, segmentation, automatic binning, and generation of cluster maps. We have thereby developed an in situ representation of the cardiac ECM that is useful for assessment of repopulation dynamics and to study the effect of local ECM composition on phenotype preservation of reseeded mesenchymal progenitor cells. This model provides a platform for studies of organ-specific cell-ECM dynamics and identification of potential cell niches.

KEYWORDS
cardiac, mesenchymal stem cells, multipotential differentiation, pericytes, progenitor cells, scaffold attachment region, stem cell-microenvironment interactions, technology

Significance statement
Stem cells reside in adult organs within specific microenvironments called cell niches. The heart is a complex organ and so far, the presence and localization of stem-/progenitor cell niches are subject to constant debate. To address these issues, the authors have developed the spatiotemporal extracellular matrix model (StEMM), which combines a modified protocol for decellularization, with cryosectioning, recellularization, and subsequent image processing including automatic binning and generation of cluster maps. The StEMM was developed within a cardiac context and validated using syngeneic mesenchymal progenitor cells. However, this model is not restricted with regard to species or organs.
1 INTRODUCTION

During embryogenesis, stem cells respond to signals from the environment and differentiate into organ-specific somatic cells. This complex interplay between cells, extracellular matrix (ECM) proteins and paracrine-/autocrine factors are crucial for organogenesis. The adult tissue retains some of the stem- and progenitor cells from development in organ-specific niches, which are specialized microenvironments defined by their location, and interaction with surrounding cells and ECM. The microenvironment maintains a balance between quiescence, self-renewal, differentiation and prevention of stem cell exhaustion, and malignant transformation. The ECM provides physical, biochemical, structural, and mechanical properties to the niche and together with bound cytokines it contributes to the control of stem cell behavior within the niche.

The stem cell niche hypothesis was first developed in the context of the hematopoietic system, where hematopoietic stem- and progenitor cells were found to reside in two different locations, the endosteal and perivascular niche (PVN), respectively. Other organs have also been demonstrated to contain stem cells in specific niches, but there is still no consensus regarding the existence of cardiac stem-/progenitor niches in the adult heart and whether these cells contribute to homeostasis and regeneration.

The heart is a complex organ, composed of highly diverse cell types of mesodermal and ectodermal origins. Hence in order to study the presence of specific stem cell-/progenitor niches in the heart, there is a need for models that are capable of representing this molecular, cellular, and anatomical complexity.

In this study, we present the spatiotemporal extracellular matrix model (STEMM), which constitutes an in situ 2D-ECM representation of the different compartments of the heart. This model allows for ex vivo recellularization with subsequent analysis through automatic binning of nuclei-segmented images and clustering by both cell and ECM markers in a spatiotemporal fashion. The STEMM is reproducible and enables studies on hundreds of tissue sections from one single heart. The model was validated by using fetal cardiac mesenchymal progenitor cells (MPCs) with characteristics of immature pericytes. The cells expressing the multipotency marker CD146 clustered in the PVN and atrioventricular junction (AVJ). Unlike the PVN, which is known to be a niche in multiple tissues, AVJ is a distinctive structure of the heart and has previously been recognized as a potential niche for cardiac progenitors. We therefore expect that the STEMM will provide a general platform for the study of cell-ECM dynamics and cell-niches. The STEMM is not restricted to the heart and can be used for studies of other organs and in different species as well.

2 MATERIALS AND METHODS

2.1 Animals

All animal experiments were performed at the animal facility of the Karolinska University Hospital in accordance with the ethical committee’s approvals D.No ID 6-17 and D.No S27-14. Hearts from male Sprague Dawley rats of age 7 to 10 weeks were used for decellularization. Pregnant Sprague Dawley female rats (gestational day [GD] 13-14) were used for isolation of the MPC fraction.

2.2 Isolation and culture of primary rat fetal cardiac MPCs

For isolation of MPCs, 10 pregnant Sprague-Dawley rats (GD 13-14) were used. The fetal hearts were removed and predigested overnight at 4°C in a 0.5 mg/mL Trypsin-solution in Hank’s balanced salt solution (HBSS). The mesenchymal fraction was prepared according to a modified version of the protocol previously developed by Laugwitz et al. The predigested heart pieces were treated for 15 minutes with 2 to 3 mL collagenase type II (Worthington Biochemical Corp, Lakewood, New Jersey) 240 U/mL in HBSS at 37°C under gentle stirring. The supernatant was centrifuged at 330 to 350g for 8 minutes and the pellet resuspended in ice-cold HBSS. The collageanase digestion was repeated until the tissue was completely dissociated. The pooled cells were washed twice and resuspended in Dulbecco’s modified Eagle’s medium (DMEM) 4.5/M199 (4:1) (GibcoBRL) containing 10% horse serum (GibcoBRL) and 5% fetal bovine serum (FBS) (PAA Laboratories Inc.) supplemented with MycoZap (Lonza, Switzerland). The adherent cell fraction (MPCs) was separated from the cardionmyocytes and endothelial cells by two rounds of culture on plastic for 1 hour each in an incubator at 37°C, 5% CO2 in 3% O2. To expand the MPC-fraction, the adherent cells were detached using TrypLE Express (GibcoBRL) and replated at a density of 10 000 cells/cm2 on cell culture treated dishes and cultured at 37°C, 5% CO2 and normoxia in DMEM high glucose (GibcoBRL), MycoZap (Lonza), Hapes (25 mM) (GibcoBRL), glutamine (2 mM) (Fisher Scientific, Sweden) and 10% FBS (PAA). When confluent, the cells were detached using TrypLE Express and washed with Dulbeccos’ Phosphate Buffered Saline (DPBS) without Ca2+ and Mg2+. A portion of the cells was frozen in Recovery-freezing medium (GibcoBRL) and stored at −180°C for later use. The remaining cells were further propagated as described above. At each passage, a portion of the cells was harvested for immunohistochemical analyses as described below.

2.3 Adipocyte, osteoblast, and chondrocyte differentiation

The MPCs were characterized with regard to their ability to differentiate into the mesenchymal lineages including adipocytes, osteoblasts, and chondrocytes using Rat Mesenchymal Stem Cell Functional Identification Kit (SC020, R&D Systems, Inc.), according to the manufacturer’s instructions with minor modifications. In short, MPCs were seeded at 10 000 cells/well in a 96 well plate precoated with gelatin type A (G2500, Sigma, Sweden). When confluent, the cells were exposed to adipocyte and osteoblast differentiation media as described in the manufacturer’s instructions and, subsequently, analyzed with immunohistochemistry. For chondrocyte differentiation,
100 000 cells were pelleted in a 15 mL falcon tube, and cultured in the chondrocyte differentiation medium as described in the manufacturer’s instructions for 21 days. After medium removal, the pellets were covered with Optimal Cutting Temperature (O.C.T.) medium (Cryomount, Histolab, Sweden), snap frozen on dry ice, sectioned using a CM1950 cryostat (Leica, Germany) and thereafter analyzed with immunohistochemistry.

### 2.4 | Smooth muscle cell and endothelial cell differentiation

The MPCs were seeded at 30 000 cells/cm² in 96 well plates. Smooth muscle cell (SMC) differentiation was performed according to a previous protocol, with some modifications. In brief, wells were coated with laminin-521 (BioLamina, Sweden) and the MPCs were seeded and maintained in DMEM/F12 w/ Glutamax containing 0.5% HI-FBS (Gibco, 11505036, Fisher Scientific), B27 (Gibco, 17504044, Fisher Scientific), Mycozap, with the addition of 10 ng/mL of PDGF-BB (PHG0044, Thermo Fisher Scientific) and 2 ng/mL TGF-β1 (PHG9214, Thermo Fisher Scientific) for 21 days.

Endothelial differentiation was carried out on laminin-coated wells: 1:1 of laminin-521 and laminin-421 (BioLamina), with M200 medium containing 1X of Large Vessel Endothelial Supplement (LVES) (M200500 and A1460801, Gibco, Thermo Fisher Scientific), Mycozap with the addition of 10 ng/mL Vascular Endothelial Growth Factor 165 (VEGF165) (293-VE-010, R&D, Bio-Techne, Sweden) for 21 days.

### 2.5 | Retrograde perfusion decellularization

The decellularization was performed using a modification of the protocol from Ott et al. We combined sodium dodecyl sulfate (SDS) and Triton X100, thereby lowering both the effective concentration and critical micellar concentration of the combined detergents. The advantage of this strategy is the improvement in decellularization and prevention of tissue-collapse while the retention of detergents is reduced.

From each rat, the lungs and thymus were gently removed before the decellularization process in order to receive an intact integral package of the ascending aorta together with the heart (n = 6). The excised hearts were stored at room temperature in 0.05 M hypotonic tris buffer pH 8.4 with 0.05 mM of EDTA (EDS, Sigma-Aldrich) containing 0.05 mM of sodium azide (S200, Sigma-Aldrich) until final dissection. For decellularization, the packages were further dissected from redundant tissues, such as remnants of the thymus, lungs, excessive fat, and connective tissue surrounding the ascending aorta. The aorta was next cannulated with a 20G IV Venflon (Becton Dickinson, BD) and attached with Ethilon sutures 6-0 (Ethicon), flushed with fresh buffer: 0.05 M hypotonic Tris buffer pH 8.4 containing 0.05 mM of EDTA and 0.05% of sodium azide, to ensure proper ligation and free flow. The hearts were attached to a custom-built stainless-steel lid and perfused with the same buffer as above overnight at a rate of 2 mL/min using a peristaltic pump (Ismatec). Next day, the buffer was changed to 0.05 M hypotonic tris buffer pH 8.4 with 0.05 mM of EDTA, 2.02 g/L of SDS (L377, Sigma-Aldrich), 530 μL/L of Triton X100 (X100, Sigma-Aldrich), and 0.05% of sodium azide, and the hearts were perfused overnight at a rate of 2 mL/min. The buffer was changed using a total of 600 mL of the decellularization buffer for a period of >72 hours. The detergents from the buffer were removed using hypotonic Tris buffer with 0.05 mM of EDTA and 0.05% of sodium azide via perfusion for >90 minutes at a rate of 2 mL/min; followed by >90 minutes of perfusion with dh20, and, finally, for >90 minutes of perfusion with DPBS (Gibco, 14190169, Thermo Fischer Scientific) containing 0.05% sodium azide for >90 minutes. The hearts were stored in DPBS with 0.05% sodium azide at 4°C, until further processing.

### 2.6 | DNA-quantification of decellularized and native hearts

Decellularized (n = 3) and native rat hearts (n = 3) were dissected and pieces of the left ventricles were weighed and subjected to proteinase K treatment. The DNA was isolated using DNeasy Blood and Tissue kit (Qiagen, Germany) and subsequently quantified using a Nanodrop Spectrophotometer DeNovix DS-11 measuring double-stranded DNA (dsDNA).

### 2.7 | Cryosectioning of decellularized whole heart

The decellularized rat hearts were subjected to stepwise sucrose infiltration (10%, 16.7%, 23.3%, and 30% sucrose in DPBS with 0.05% sodium azide). In each step, the hearts were incubated for >1 hour before they were transferred to the next step. After sucrose infiltration, the hearts were immersed in a 1:1 mixture of O.C.T. and 30% sucrose in DPBS with 0.05% sodium azide for 30 minutes before embedding in O.C.T and snap freezing at −80°C. Cryosections of 10 μm thickness were generated using a CM1950 cryostat (Leica, Germany) and transferred to empty wells of room temperature six-well cultures plates (TPP, Techno Plastic Products). The tissue sections were allowed to melt and dry at room temperature before storage at 4°C.

### 2.8 | Culturing of cells on decellularized heart cryosections with subsequent fixation and nuclei staining

Prior to cell seeding, six-well plates containing the tissue sections were incubated overnight in DPBS supplemented with 0.05% of sodium azide (3 mL/well) to prevent pathogenic growth. After incubation, the wells were washed three times with DPBS and incubated with 3 mL cell culture medium (DMEM Low Glucose with GlutaMAX and with phenol red; 10% HI-FBS; 1X Mycozap) per well for >30 minutes while preparing cells for seeding. The MPCs were incubated with TrypLE (Gibco,12604039, Thermo Fisher Scientific) for
5 to 10 minutes, centrifuged at 500g for 5 minutes and resuspended in culture medium. The cells were seeded at a density of 100,000 cells/well in 3 mL medium per well. The plates with the cells were incubated at 37°C with 5% CO₂ in normoxia for 2 hours, followed by a washing step to remove unattached cells, and subsequent addition of fresh medium.

The sections with cells were fixed using 80% methanol (~0.2 mL medium and 0.8 mL methanol) for 10 minutes at room temperature or for 10 minutes at 37°C in the incubator. The methanol was replaced with 3 mL of DPBS with 0.05% sodium azide per well. Once all wells in a plate were fixed, the cells were stained with 4',6-Diamidino-2-Phenylindole (DAPI) (D2149, Invitrogen) in PBS (09-8912-100, Medicago) for 10 minutes, washed three times with PBS for 10 minutes each time, and imaged as described below.

2.9 | Immunohistochemistry

Sections for in situ cultures were stained with DAPI and subjected to imaging prior to antibody staining or hematoxylin and eosin (H&E) staining. All other immunohistological samples were counterstained with DAPI after antibody staining. The in situ and in vitro cultures were fixed in 80% methanol with 20% medium. All other immunohistological samples were fixed in 80% methanol with 20% dH₂O. All samples were washed three times 10 minutes in PBS and blocked with 5% serum (goat serum, PCN5000, Fisher Scientific; donkey serum, ab7475, Abcam) in PBS for 30 minutes at room temperature, then incubated overnight at +4°C with primary antibodies at a dilution of 1:200 in blocking solution followed by four 10 minutes washes with PBS. Secondary antibody was then added at a dilution of 1:700 in blocking buffer and incubated for 1 to 3 hours at room temperature, followed by final washing with PBS three times 10 minutes.

2.10 | Antibodies

The following primary and secondary antibodies were used: Apelin receptor (APLNR) (Thermo Fisher Scientific Cat# 702069, RRID: AB_2633058), PDGFR-α (Abcam Cat# ab96569, RRID: AB_10687154), Periostin (Abcam Cat# ab14041, RRID:AB_2299859), CD31 (Abcam Cat# ab64543, RRID:AB_1141558), CD45 (Abcam Cat# ab33923, RRID:AB_726543), CD90 (Abcam Cat# ab225, RRID:AB_10672212), CD105 (Abcam Cat# ab2529, RRID:AB_303134), CD73 (Abcam Cat# ab133582), CD146 (Abcam Cat# ab75769, RRID:AB_2143375), NG2 (Abcam Cat# ab83178, RRID:AB_10672215), PDGFR-β (Abcam Cat# ab32570, RRID:AB_777165), VE-cadherin (Abcam Cat# ab33168, RRID:AB_870662), Calponin/CNN1 (Abcam Cat# ab700, RRID:AB_305697), Collagen 1 (Millipore Cat# 234167, RRID:AB_2074634), Laminin α5 (Thermo Fisher Scientific Cat# PA5-49930, RRID:AB_2635383), VEGF (Abcam Cat# ab46154, RRID:AB_2212642), and Histone 3 phospho S10 (Abcam Cat# ab14955, RRID:AB_443110). Aggrecan (967800, RnD), FABP-4 (967799, RnD), and Osteocalcin (967801, RnD) from the RnD kit (SC020, RnD), Goat-anti-Mouse (Thermo Fisher Scientific Cat# A-11004, RRID:AB_2534072), Thermo Fisher Scientific Cat# A-21121, RRID:AB_2535764), Goat-anti-Rabbit (Thermo Fisher Scientific Cat# A10040, RRID:AB_2534016), Donkey-anti-Rabbit (Abcam Cat# ab150061, RRID:AB_2571722), Donkey-anti-Mouse (Thermo Fisher Scientific Cat# A-21202, RRID:AB_141607), Donkey-anti-Goat (Abcam Cat# ab175704, RRID:AB_2725786), and Donkey-anti-Rabbit (Thermo Fisher Scientific Cat# A10040, RRID:AB_2534016).

2.11 | Microscopy and image acquisition setup

Images of the H&E-staining, in situ, and in vitro cell cultures were acquired with Olympus IX73 inverted microscope using objectives UPlanFLN 4x (17 mm WD, NA 0.13, PHL) and UPlanFLN 10x (10 mm WD, NA 0.30, Ph1), with filter cubes detecting DAPI, Fluorescein isothiocyanate (FITC), and Tetramethylrhodamine (TRITC) respectively. The fluorescence images were taken with an exposure time of 1 second with 2x digital gain. When phase contrast images were acquired, the exposure time was manually adjusted. Images displaying MNC differentiation into adipocytes, osteoblasts, and chondrocytes were acquired with confocal microscope LSM 700 AxioObserver (Zeiss). Using objective Fluor 10x/0.50 M27 and excitation lasers 405, 488, and 555 with corresponding detection wavelength 420 to 480, 300 to 630, and 560 to 800, respectively. One channel was acquired at a time with pinhole size ~1 AU, at core facility BioVis, Uppsala University. Exposure time and gain were set below pixel saturation visualized within the software's live view in the acquisition window. Samples with secondary antibody only (technical negative control) had the same exposure time and pinhole settings as positive samples.

2.12 | Preprocessing of images with ImageJ

The phase contrast images were opened and processed in FIJI (Fiji Is Just ImageJ) distribution package of ImageJ (Fiji, RRID:SCR_002285). .vsi-files were read with the plugin “OlympusViewer” (Olympus Corporation). Images captured before and after staining were opened and stacked together. Since OlympusViewer automatically normalizes the intensities across all images, the image with the highest intensity was opened first. We used the “BaSiC” plugin to remove vignette effects on the images. Images captured before and after staining were adjusted for vignette simultaneously. For images acquired with the DAPI channel, only the images acquired before staining were needed to be processed for vignette effect. The corresponding field of view of phase contrast images was opened in the same order as the channels of interest; each channel had different order of opened images, prioritizing the image with the highest intensity. Contrast and contours of the phase contrast images were subsequently enhanced through the “find edges” function followed by stitching. The stitching generated a .txt-file with coordinates of the same order as the images in the folder that was stitched. As such, the corresponding field of view images from the fluorescence channels could be stitched.
Characterization of cultured and differentiated rat fetal cardiac mesenchymal progenitor cells. Cells derived from third trimester fetal rat hearts expressed several markers characteristic for pericytes: abundant expression of (A) APLNR, PDGFR-β, CD146, NG2; lower expression of CNN1 and VE-cadherin; only a few cells showed the expression of CD90 and none showed expression of CD31, CD45, CD105, and PDGFR-α. The cells were able to differentiate into the mesenchymal lineage, which included bone, fat, and cartilage, defined by their respective expression of Osteocalcin, FABP-4, and Aggrecan. (C) The cells were also able to differentiate into smooth muscle cells with increased expression of CNN1 and (D) to cells with endothelial progenitor properties, defined by VE-cadherin, but no CD31 expression. Nuclei were visualized by 4',6-Diamidino-2-phenylindole (DAPI) staining (magenta) and specific antibody (green). Scale bars = 100 μm.

APLNR, apelin receptor; CNN1, calponin; FABP-4, fatty acid binding protein-4; NG2, neural/glial antigen 2; PDGFR, platelet derived growth factor receptor; VE-cad, vascular endothelial-cadherin.
together according to the coordinates using stitching again, with the option “Coordinates from file.” Images acquired before and after staining were stitched together and thus automatically aligned. To separate images captured before and after staining and generate tiled images, images of the unstained samples were set to brightness = 0 and stitching was performed with “Max Intensity” as a setting for the merging calculations. After that, the same procedure was performed for the images of the stained samples and set to brightness = 0, while unstained images of the samples were kept as they were. All subsequent image calculations were then performed in the R software.

2.13 | Image processing with R

An R script is provided in the Supporting Information (Supplementary R code). In short, images from each channel were read into R (R Project for Statistical Computing, RRID:SCR_001905) using the EBImage package. The ratio was then calculated between the modes of tiled images of samples before and after staining, multiplying the images of stained samples with the calculated ratio. This was done under the assumption that background noise was the source for the majority of signal in the images. The images of stained samples were then subtracted with the unstained images of the samples, removing the majority of background and auto fluorescence signal. For the DAPI channel, subtraction was performed using the FITC filter from the same image. This yielded an intensity histogram similar to one-half of a normal distribution. Extending the graph by mirroring the histogram and adding it to existing data, a bell-shaped histogram could be generated. Based on this new histogram, the SD could be estimated. We assumed that the majority of the data was from the background and low-intensity boundaries of the nuclei. We therefore set a threshold of 2 SDs for our original data of the DAPI channel, which generated a binary image of the nuclei that could be used for segmentation.

3 | RESULTS

3.1 | Syngeneic fetal cardiac-derived MPCs to be used in the StEMM

To validate our approach, we used MPCs with pericyte characteristics that are known to reside in the PVN and are found in several organs. Thus, the MPCs can be regarded as a representative and relevant cell type for this study. The cells were isolated from third trimester hearts of Sprague-Dawley rats, and demonstrated low expression of CD90, and the demonstrated differentiation capacity (Figure 1), collectively identify the cells as MPCs with pericyte characteristics.

3.2 | Decellularized whole heart provides ECM and anatomical mapping as substratum in the StEMM

In order to assess the dynamics between progenitor cells and a preserved complex ECM, we developed the StEMM, which allows for analysis of inter- and intrasample phenotypes of cell populations in a multiplexed manner, through clustering and visualization.

We decellularized whole hearts through retrograde perfusion (Figure 2A). In order to preserve key components of a healthy ECM, we modified the decellularization method introduced by Ott et al., by combining SDS and Triton X100 into one step. Decellularization was confirmed with the absence of intact nuclei (Figure 2B) and reduced dsDNA content (Figure 2C).

After decellularization, the hearts were subjected to stepwise sucrose infiltration, embedding, and cryosectioning, which generated 10 μm thin matrices to be used for recellularization. The sections successfully attached to wells of tissue-culture plates. Immunohistochemistry demonstrated that the major ECM components were retained (Figure 2B,D), including structural fibrillary collagen I, chains of the major basement membrane component laminin α5 (LAMAS), found in both the adult and developing heart, and peristin (POSTN), which is an important mediator during cardiac valvular development and in the adult heart (Figure 2D). The paracrine factor VEGF was also present after decellularization (Figure 2E,F), which is known to be bound by the ECM, demonstrating that the StEMM not only preserves basal membrane proteins, but also factors bound to the ECM.

To ascertain that the tissue sections could function as a substrate for cell adhesion and growth, we seeded the MPCs in the wells containing the sections (Figure 2B) and upon incubation at normoxia and 5% CO2, the cells successfully attached and proliferated on the ECM matrices (Figure 2B).
FIGURE 2  Legend on next page.
Decellularization retained important extracellular matrix (ECM) components and enabled 2D recellularization. Excised rat hearts were subjected to retrograde perfusion decellularization. A, A visual representation of the decellularization process at different time points, where cellular components were sequentially removed, yielding a final translucent ECM heart structure. B, Upper panel, 4',6-Diamidino-2-phenylindole (DAPI) and hematoxylin-eosin stainings (H&E) of the same sample demonstrate absence of nuclei in the decellularized heart sections. The lower panels demonstrate the presence of nuclei with both DAPI- and H&E-stainings, after 48 hours of culturing of mesenchymal progenitor cells (MPCs) on a consecutive section. C, Reduced double-stranded DNA (dsDNA) after decellularization, bar plot of the mean and SD. D, Important ECM structures were preserved, which is demonstrated by the presence of collagen 1, laminin α5, and peristin. E, Retention of VEGF after decellularization, compared to (F) the native heart. In the figure, first column: phase contrast edges; mid-column: specific antibody; last column: a combination of both. Specific antibody in green and phase contrast edges in magenta. Scale bars in (B) = 100 μm, scale bars in (D) = 1000 μm, scale bars in (E) and (F) = 200 μm. COL1, collagen 1; HE, hematoxylin and eosin; LAMA5, laminin α5; PH edges, edges of phase contrast; POSTN, peristin; VEGF, vascular endothelial growth factor

### TABLE 1 Algorithm for automatic binning of a nuclei segmented image

| Input: Segmented nuclei image as a matrix |
|------------------------------------------|
| Procedure (Pseudocode):                 |
| store mean nuclei diameter as a starting bin width |
| while the current number of bins containing more than 1 cell is greater than in the previous step { |
| increase current bin width by the mean diameter |
| create a temporary matrix to store the number of cells in the bins with a size based on the original matrix divided by current bin width |
| for each step row wise { |
| for each step column wise { |
| Extract sub matrix of the original matrix, with the current bin width. Compute the number of cells in this sub matrix by counting the number of unique digits in it. |
| if number of unique digits is greater than 0 { |
| for each cell in the sub matrix { |
| compute the fraction of each cell's area |
| } |
| } |
| sum all the fractions to get the total number of cells in sub matrix |
| store the cell density for current sub matrix |
| calculate number of bins containing more than 1 cell |
| } |
| } |
| } |
| Output: Optimal bin size |

#### 3.3 An algorithm for automatic binning enables generation of density maps

In order to facilitate the analysis of the StEMM, we developed an algorithm for generating density maps based on the nuclei densities generated from seeded cells. When analyzing histological sections, the bin size for the image is usually chosen manually and arbitrarily, which impairs reproducibility between different experiments. To address this issue, we developed an algorithm that automatically identifies an optimal bin size based on the maximal number of bins containing at least one cell (Table 1). The algorithm was based on the Nyquist theorem,52 which, in short, means that the sampling rate should be twice that of a signal's measured bandwidth, in order to fully resolve the signal without the loss of information at a minimum sampling rate. In our case, the nucleus represents the signal of interest and the diameter represents the signal's bandwidth, where one nucleus in its simplicity represents one cell. Hence, the mean nucleus diameter is set to the initial bin width and will therefore serve as the starting point for the automatic binning process of down sampling the resolution that continues until the single nuclei are unresolved, for the generation of density maps.

According to our algorithm, the bin width increases incrementally by one mean nucleus diameter per iteration until the algorithm has found the optimal bin size. As a consequence, with increasing bin width the overall number of bins will decrease (Figure 3A). The iterative process of the algorithm pools fractioned and single cells and generates multicellular bins that increase in number until reaching a maximum, followed by an exponential decrease (Figure 3B). Hence, the relationship of bin width and number of multicellular bins yields a nonlinear relationship with an initial rise and subsequent drop, where the peak of the curve represents the maximum number of bins that includes at least one cell (Figure 3B). The fraction of bins containing more than one cell increases rapidly until the peak has been reached, which is followed by a much slower increase (Figure 3C). This is due to the loss in resolution when areas containing no cells are steadily binned together with areas containing multiple nuclei. Interestingly, during the earlier culturing time points of 8 hours up to 48 hours, the cell nuclei were larger than at the later time point of 96 hours (Figure 3D,E). Consequently, larger bin width was needed to capture at least one cell per bin during earlier cultures in comparison to later time points, implying an advantage of using automatic binning due to the sample variations.

#### 3.4 Clustering of multiplexed density maps mediates discovery of regions of interest in the StEMM

The StEMM was used to generate and analyze images in a spatiotemporal manner, where temporal resolution was given by fixation of the cultured MPCs on sections at 8, 48, and 96 hours; and compared to a negative control with no cells (Figure 3). Intrasample normalization was performed by calculating the ratio of all bins in the sample by the bin area (eg, pixel H * pixel W), with a unit of cells per pixel. Similarly, the intersample normalization was calculated by dividing the bins from...
all samples with the maximum bin value found in all samples. Thereaf-
	er, gap statistics on k-means clustering were used to determine the

optimal number of clusters needed to generate well-separated cluster

data from the density maps of one (CD146) and two markers (CD146

and nuclei), respectively (Figure 3F). However, no clear optimal num-

ber of clusters could be identified from the gap statistic method.

FIGURE 3

The algorithm for automatic binning generated a nonlinear relationship
between the numbers of bins containing more than one cell and k-means clustering in
the StEMM. The mesenchymal progenitor cells (MPCs) with pericyte characteristics
were cultured for 8, 48, and 96 hours. The matrix without cells was used as a negative
control. A, The total number of bins as a function of increasing bin width. B, Number
of bins containing more than one cell. C, The fraction of bins containing more than one

cell. D, The distribution of nuclei sizes between the different samples, illustrated by
a violin plot combined with a boxplot. E, Histogram of the distribution of nuclei sizes
between the different samples. Cell density maps were then made from nuclei- (4',6-
Diamidino-2-Phenylindole, DAPI) and CD146 stained samples and analyzed (F-J). F, Gap-
statistics of k-means for each density map alone and in combination. G-I, k-means
clustering with clusters ranging from 2 to 64, with increments of the power of two. G,
Clustering based on nuclei density. H, Clustering of CD146 density and (I) clustering of both CD146 and nuclei. J, Scatterplots of increasing number of clusters for the combination of density maps. Scale bars = 1000 μm. StEMM, spatiotemporal extracellular matrix model.
Instead, representative clustered images were generated based on the increasing number of clusters (Figure 3G-I). As shown in Figure 3G, at 8 hours the cells (nuclei) were equally distributed over the different compartments of the heart, regardless of the number of clusters that were studied. At 48 and 96 hours of culture, the distinct region of the AVJ demonstrated an increased number of nuclei, which was most evident when two and four clusters were studied (Figure 3G). CD146 also clustered to the same region at 96 hours of culture, which was optimally assessed using 2 to 32 clusters (Figure 3H). The clustering characteristic was preserved when nuclei and CD146 data were combined, with the difference that the clustered regions were prominent independently of the number of clusters that were used (Figure 3I). According to the scatter plot, the discrimination between nuclei and CD146 was best studied when 16 to 32 clusters were used (Figure 3J).

Based on the clustering data, CD146 and nuclei were mainly clustering at the AVJ. This was next confirmed by extracting this region of interest (ROI) from the fluorescence images (Figure 4A-C), and superimpose the edges of phase contrast (Figure 4D). This confirmed that the region demonstrating clustering of CD146 and nuclei corresponded to CD146+ cells (Figure 4). The CD146+ cells in the AVJ were identified in two different locations. One area was found to be in the left atrial wall, proximal to the mitral valve and dorsal to the aortic root (Figure 4D*) and the second area was located in the perivascular region of the ventricular wall, close to a marginal branch of the circumflex artery (Figure 4D**).

### 3.5 Multiplex analyses of extracellular and cellular markers in the StEMM highlight their spatial correlation

The relationship between cell densities and clustering of PDGFR-β or CD146 in relation with LAMA4 was studied in decellularized whole heart sections (Figure 5). CD146 with nuclei and LAMA4 clustered mainly in the main arteries and at the AVJ (Figure 5A). PDGFR-β showed distinct clustering with nuclei and LAMA4 throughout the whole heart, which is consistent with the findings from the immunofluorescence images (Figure 5B). Accordingly, cells with high expression of PDGFR-β and low CD146 expression were mainly found in the peripheral parts of the heart, while the cells with high PDGFR-β and CD146 expression were mainly found at the aortic root and AVJ (Figure 5). Cells attaching to the surrounding plastic outside the ECM clustered together and showed a relatively low expression of both CD146 and PDGFR-β, as observed in the immunofluorescence images (Figure 5A,B).

### 3.6 Comparison between specific regions using the StEMM and the native rat heart

To test the ability of the StEMM to recapitulate the in vivo regional features of an organ, we immunostained similar ROIs in the native rat heart (Figure 6). CD146+ cells were found as a cluster in the center of the AVJ (Figure 6A, dotted region and arrow), with a clear overlap...
of LAMA4, observed in a separate staining (Figure 6C, dotted region). PDGFR-β was not apparent in the AVJ (Figure 6B, dotted region), instead prominent expression was evident in the valves, valvular base and the PVN (Figure 6B, dashed circle). Since LAMA4 was relatively diffusely spread with varying staining intensities in the native heart as well as when using the STEM, we also analyzed the expression of other major ECM components (LAMA5, COL1, and POSTN). The basement membrane protein LAMA5 was extensively stained in the heart (Figure 6D), with slightly higher intensity in the PVN (Figure 6D, dashed circle) and less in the interstitial valvular root, including the left and upper parts of the AVJ (Figure 6D, dotted region). POSTN on other hand was abundant in valves and stained as thin streaks reaching into the AVJ (Figure 6E, dotted region). Finally, COL1 was abundantly present in the PVN (Figure 6F, dashed circle) and in the valves, but not in the AVJ (Figure 6F, dotted region). Based on these distribution maps, PDGFR-β was found to be expressed in the regions also positive for LAMA4, LAMA5, POSTN, and COL1. In contrast, CD146 was linked to the presence of its ligand LAMA4.
The main objective of this study was to develop a model for in situ studies of cell-ECM dynamics that we demonstrated to be useful for identifying potential cell niches of the heart. We did so by generating sections of decellularized whole hearts, in which all anatomical regions were represented, onto which syngeneic fetal cardiac MPCs were seeded. In order to study the expression of cell surface markers and spatial distribution of cells over time, we developed an algorithm that automatically bins nuclei segmented images and generates density maps in the StEMM. Interestingly, when collating density maps from histological sections, the bin size is often set manually in the range of 50 to 150 μm.\textsuperscript{54-56} In contrast, by the use of our algorithm, bin size was automatically set between 45 and 115 μm. This reduces the risk of losing data since binning depends on both cell size and distribution, which in turn is dependent on organ type and mass.\textsuperscript{57}

**FIGURE 6** Region of interest identified by the StEMM is also present in the native heart and partly defined by laminins. The region of interest which was identified in the StEMM was confirmed by immunohistochemistry of native rat hearts: (A) CD146 expression was clustered in the middle of the AVJ region (dotted region), as well as in the PVN (dashed circle). B, PDGFR-β was also expressed in the PVN, in the aortic valves and aortic root, but not in the AVJ. C, LAMA4 was more generally expressed in the heart, with predominance in the AVJ. D, LAMA5 was also found in all parts of the heart, but with no predominant regions. E, POSTN was identified in the aortic valves and root with thin streaks into the AVJ. F, The aortic valves and root together with the PVN also stained positive for COL1, but not the AVJ. In the figure: scale bars = 200 μm. AVJ, atrioventricular junction; COL1, collagen 1; LAMA4, laminin α4; LAMA5, laminin α5; PDGFR, platelet derived growth factor receptor; POSTN, periostin; PVN, perivascular niche; StEMM, spatiotemporal extracellular matrix model.
During the development of the StEMM, the main focus was on the log- and lag-phases of a well-defined MPC culture, where the cell density and cell marker expression of CD46 in culture of periods between 8 and 96 hours was compared, thus capturing the association of ECM composition with cellular phenotypes and thereby identify regions of interest. Surface manipulation of decellularized ECM using exogenous matrices or specific enzymatic removal of endogenous molecules alters cellular responses during culture.\textsuperscript{58,59} Retaining native complex matrices after decellularization is therefore of great importance for such studies, where not only the gross macrostructures need to be preserved, but also basement membrane proteins and bound paracrine factors and cytokines. In our StEMM methodology, we demonstrated effective decellularization by removal of nuclei and dsDNA with retained representative components of the ECM like COL1 and LAMAS, and also the presence of VEGF, which is known to bind a variety of ECM molecules. Along with an intact anatomical macrostructure, and by using a progenitor cell with pericyte characteristics, changes in surface expression of markers could be used to identify the ROI and possible niche regions.

The MPCs were cultured on the decellularized heart sections containing a retained complex ECM and during a culture period ranging from 48 to 96 hours, the MPCs expressing CD146 clustered in both the AVJ and in the PVN, whereas PDGFR-\(\beta\) cells demonstrated a more widespread distribution pattern that was similar to that of laminin \(\alpha4\) chain.

In the native rat heart, CD146\(^+\) cells mainly resided in the AVJ and occasionally in the PVN, while PDGFR-\(\beta\) cells on the other hand were more prevalent in the PVN, while absent in the AVJ. These differences in the distribution of PDGFR-\(\beta\) and CD146\(^+\) cells were also captured using the StEMM. As shown by the StEMM, laminin \(\alpha4\) was strongly stained in the AVJ, while the other ECM components displayed different distributions. The similarities in the markers' distribution patterns validate the use of StEMM as a relevant in situ model of organs and tissues.

The presence of niches of stem-/progenitor cells in the heart, as well as the role of these cells for heart regeneration, is still a matter of debate. We do know that there is a constant turnover of cardiomyocytes during the whole life span,\textsuperscript{60} but whether these cells are generated from progenitors from certain niches or from proliferation/differentiation of noncardiomyocytes or cardiomyocytes is still questionable. Furthermore, there is no consensus regarding the phenotypic characteristics of a cardiac progenitor. Several groups have identified different potential cardiac progenitors but whether these cells really contribute to the regeneration of the heart needs further investigation.\textsuperscript{61,62} As these issues warrant clarification, the StEMM might provide a model to test different potential progenitor cells, concerning their location and association with different ECM components, and thereby aid to characterize niche regions. Upon identification of these regions using the StEMM, the corresponding regions in native hearts can be determined and characterized further.

As the StEMM is not restricted to the heart, this approach can be used for other organs in order to assess potential niche compositions and their respective progenitor cells. In comparison to other approaches, the StEMM preserves the complexity and the macrostructure of the ECM and allows for a large number of samples to be generated and analyzed simultaneously. The frequent use of recombinant proteins to mimic the ECM does not reflect the complex composition of the matrix, and the use of homogenized ECM\textsuperscript{63,64} destroys the macroscopic structures, while the 3D de-/recellularized tissue models\textsuperscript{65-67} are too labor- and time-consuming to enable concomitant analyzes of multiple samples. The StEMM on other hand enables semi-high throughput use of standard wide-field microscopy of an in situ 2D cross-sectional representation of the whole heart's ECM. As the sections have a thickness of 10 \(\mu\)m, they can sufficiently capture a quasi-3D complex composition of the ECM molecules given that one of the largest ECM components, collagen 1, has a characteristic length of about 300 nm.\textsuperscript{68}

The main limitation of the StEMM is, in our view, the need to compensate for autofluorescence by manual calculations. Autofluorescence is an inherent problem during tissue imaging. However, if all samples undergo identical processing, the impact of autofluorescence should be regarded as a systematic error that can be corrected. Another limitation is the biased choice of ECM components analyzed in the current study. Optimally, the presence of a wide range of basement membrane proteins and other ECM components should be evaluated. However, in this study we focused on the large and heavily crosslinked fibrillary collagen 1, the noncovalently bound laminins containing \(\alpha4\) and \(\alpha5\)-chains, and the smaller sized periostin, all with distinct distributions and functions.

## 5 | CONCLUSION

We have demonstrated that the StEMM provides a platform that facilitates reproducible semi-high throughput studies of cell-ECM dynamics and cell-niche identifications. In this study, we used the heart as a model organ, but the concept of StEMM can be applied to other species and organs as well.

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## CONFLICT OF INTEREST

K.-H.G. and S.R. are co-owners of the company IsletOne AB. The other authors declared no potential conflicts of interest.

## AUTHOR CONTRIBUTIONS

K.O.: conception and design, methodology (programming, cell culture, decellularization), collection and assembly of data, data analysis and interpretation, writing—original draft, final approval of manuscript; S.R.: methodology (experimental design of cell culture), data interpretation, writing—editing/review, final approval of manuscript; W.C.M.: methodology (experimental design of decellularization), writing—editing/review, final approval of manuscript; C.O.: methodology
DATA AVAILABILITY STATEMENT

Resource availability: Further information and requests should be directed to the Lead Contact. Karl-Henrik Grinnemo (karl-henrik.grinnemo@surgsci.uu.se). Materials availability: There are restrictions to the availability of cells due to the lack of an external centralized repository for its distribution and our need to maintain the stock. Data and code availability: The published article includes the code for the algorithm used in this study (Supplementary R code).

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