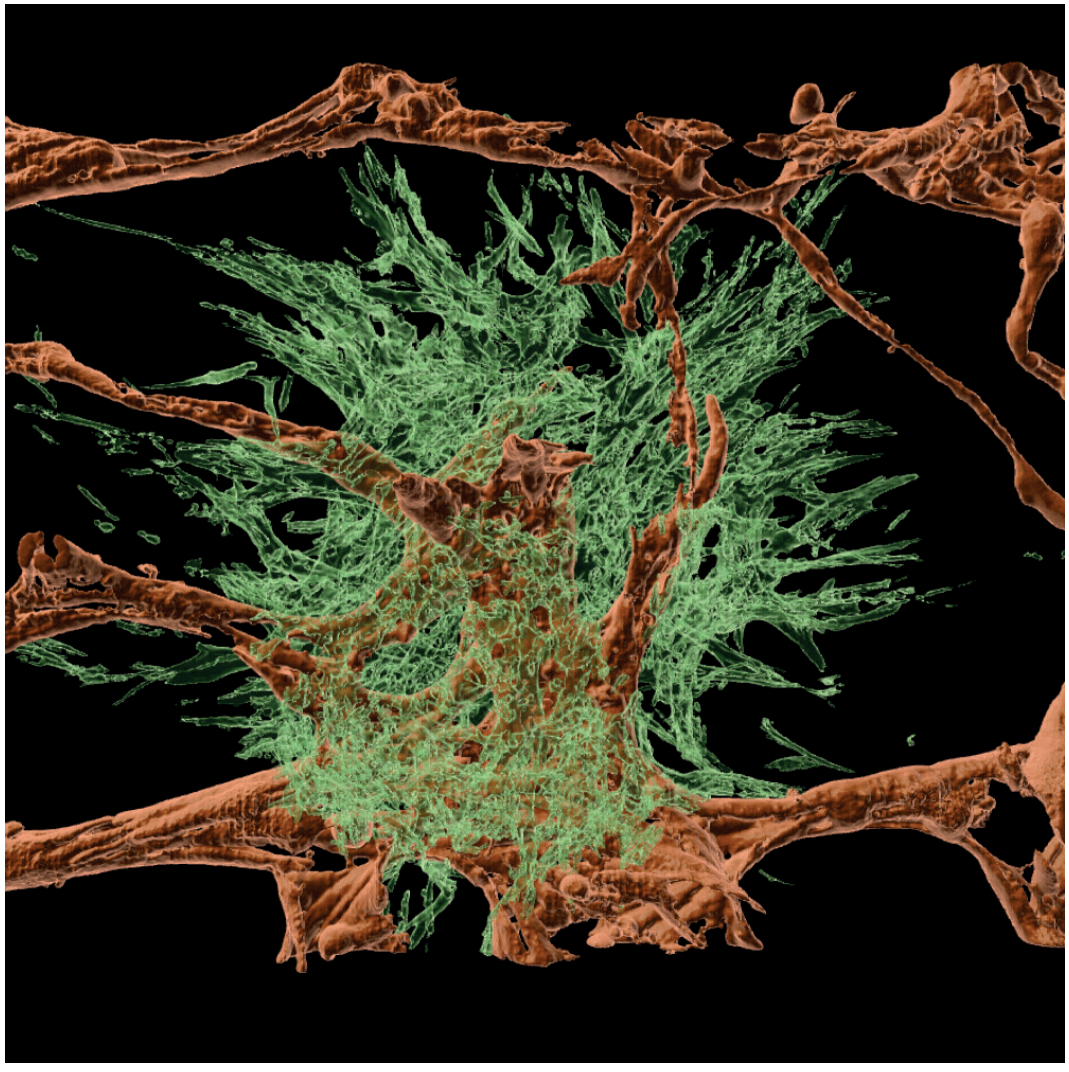
# hiPSC-derived 3D cardiac microtissue models with integrated immune cells and vasculature

Arslan-van Bergen, U.

# General Discussion



*3D reconstruction of VMToC showing lumenized microvascular network formed by hiPSC-ECs (mCherry, orange) embedded in hiPSC-CMs (ACTN2, green).*

## General discussion

In this thesis we developed hiPSC- derived 3D cardiac tissue models integrating various cardiac cell types and other important components such as immune cells and functional vasculature. We defined optimal medium conditions for the incorporation of immune cells, specifically macrophages within the microtissues. Notably, culture plate type was important to keep macrophages inside the microtissues and prevent them migrating out; the microenvironment of microtissues also exerted an influence on the phenotype that macrophages acquired.

Besides immune cells, by introducing endothelial and mural cells in an microfluidic organ-on-chip platform, we facilitated the self-organization of lumenized external vessels around microtissues through which fluid could flow. The endothelial cells in the microtissues were able to link with external vasculature in the microfluidic chips forming a continuous vascular network and became perfusable under flow. This was evidenced by the passage of small fluorescent beads through the synthetic vasculature. These models offer opportunities to recapitulate more complex human cardiac microenvironment, study cardiac cell dialogue and pathophysiology of disease, to enable discovery of new strategies for understanding disease mechanisms and supporting drug development.

## Current 3D Cardiac Models

Since the initial discovery of human embryonic and induced pluripotent stem cells (hESC or hiPSC, collectively called hPSC), they became a renewable cell source which could differentiate into all cells of the body (Thomson, 1998; Takahashi et al., 2007, 2006). hESCs were originally differentiated in aggregates called embryoid bodies, which when exposed to different stimuli or growth factors, could directed to form derivatives of different germ layers. However, this was at low efficiency. Much effort was placed in converting these protocols to high differentiation efficiency, 2D models using defined factors and culture medium without fetal calf serum as supplement. These protocols were effective in both hESC and hiPSC. hPSC-derived 2D models are valuable to give insights into the phenotypes of specific cell types. However, they essentially failed to answer questions related to organ development and (patho)-physiology. This was mostly because they missed key components of the real organ and did not show the same functional cellular maturity or crosstalk between cells. Going back once more to better defined hPSC-derived 3D models, has provided new perspectives and these are now regarded as holding significant potential for truly recapitulating their *in vivo* counterparts and modelling complex disorders more accurately. In **chapter 2**, we reviewed current state-of-the- art 3D cardiac models that can integrate multiple cell types, chemical and mechanical microenvironmental cues. In this short review, we reflected on their current use and perspectives on their future utility as cardiac models. In **chapter 3**, we discussed and compared these 3D cardiac models. We reviewed in detail their applications now that maturation methods are improving, allowing better human disease modelling, study of development and screening of drugs. We focused on the structural and functional readouts that are current practice, highlighting the more non- invasive options. Finally, we discussed future perspectives for their implementation both in industry and academia.



## Establishment of 3D Cardiac Microtissue that Integrates Immune Components

Central to much of this thesis, has been the use of cardiac microtissues, developed in our lab to better model the heart. Upon starting the research described here, protocols had been established to generate hiPSC-derived cardiomyocytes (CM, Giacomelli et al., 2020), endothelial cells (EC, Giacomelli et al., 2017; Orlova et al., 2014) and cardiac fibroblasts (CF,

Giacomelli et al., 2020). By integrating CMs with vascular and stromal cells, it had been shown CM maturation could be achieved, at least to a post-natal stage (Giacomelli et al., 2020). In 2D culture, CMs only reach developmental stages equivalent to mid-gestation. Several other groups also developed 3D cardiac models that mainly focus on these three cell types as they represent the key components of the cardiac microenvironment and their interactions play crucial roles in maintaining homeostasis in health and disease further (Beauchamp et al., 2020; Mills et al., 2019; Richards et al., 2020). However, immune cells which are the underlying trigger of many diseases, including several of the heart like myocardial infarction, are important for creating more comprehensive and relevant models. Our group had previously induced hiPSC differentiation to immune cell types such as monocytes and macrophages (IPSDMs) (Cao et al., 2020). Interestingly, these IPSDMs have higher efferocytosis and endocytosis activity compared to blood-derivatives which suggests their resemblance to yolk-sac derived cardiac resident macrophages (CRMs, Cao et al., 2019). Yolk-sac derived CRMs are mainly involved in regeneration and angiogenesis and they can self-renew (Epelman et al., 2014; Dick et al., 2019; Bajpai et al., 2018). By contrast, postnatally, they lose their capacity for self-renewal and are replaced partially by blood-circulatory monocytes which are more involved in pro-inflammatory processes (Epelman et al., 2014; Bajpai et al., 2018). This switch in the immune population in the heart determines its response to inflammatory stimulus like injury.

In an effort to address questions related to IPSDM phenotype in tissue microenvironment, in **chapter 4**, we used IPSDMs differentiated according to the established protocols, and optimized conditions to integrate them into 3D cardiac microtissues together with CMs, ECs and CFs. In our first attempts, we encountered several problems: (1) macrophages migrated outside of microtissues; (2) they attached to culture surface of the wells; (3) the number of macrophages inside the microtissues were not very high at the end of 21 days culture period (Chapter 4, Figure 1).

Towards solving these problems, we first optimized the plate type. Following our previous microtissue protocol, we initially used a standard V-bottom plates to generate microtissues with IPSDMs (CMECFM0) in which IPSDMs migrated outside and attached to the wells. As a result, at the end of 21 days culture period, the number of IPSDMs inside CMECFM0s were very low. Surprisingly, the numbers seemed to increase upon longer term culturing (31 days). However, to date, we do not know the effect of longer culture periods on other cell types, especially on CMs. Therefore, we did not continue with microtissues that were cultured longer than 21 days.

Our first hypothesis for the migration problem was the increased cell death in the V bottom plates. After seeding the cells in these plates, centrifugation is necessary to form a compact microtissue. However, also because of the centrifugation, microtissues become stuck to the bottom of wells which might increase cell death. As a result, we believe the IPSDMs are actually attracted to the dead cells and this could be the reason for migration. To decrease the cell death, microtissues were instead generated using standard U-bottom plates; in this case, the microtissues formed but did not stick to the bottom of the plates. However, IPSDM

migration and attachment to the wells was still evident in this plate type, suggesting that the first hypothesis on attraction to dead cells was incorrect (Chapter 4, Figure 2B-D). We then tried ultra-low attachment U-bottom plates which eliminated both the migration and attachment problems. In these plates, IPSDMs remained inside the microtissues and microtissues showed compact formation (Chapter 4, Figure 2E-G). Furthermore, in these plates, the boundaries of microtissues were intact and "clean". This was different from the microtissues without IPSDMs where there was usually a halo of cells present around the microtissue. This possibly indicates an efferocytosis of IPSDMs in microtissues. However, to confirm this hypothesis, further tests, such as live-dead imaging of microtissues with and without IPSDMs, should be done.

Once the most appropriate protocol had been identified, we next characterized the effect of culture conditions on different cellular subsets. We showed that macrophage colony stimulating factor (M- CSF) significantly increased the IPSDM number in microtissues in earlier culture periods (Chapter 4, Figure 3). This confirms a previous report on the effect of M-CSF on survival and proliferation of IPSDMs (Otero et al., 2009). Interestingly, IPSDMs numbers on day 21 microtissues with and without M-CSF were similar and overall IPSDM numbers decreased over the 21 days culture period (Chapter 4, Figure 4A). This apparent discrepancy could be partly explained by the culture medium. Here, we used B(P)EL medium with growth factor supplements. This medium had previously been used to culture the microtissues (Campostrini et al., 2021), but not for the IPSDMs. Switching to a medium that can support IPSDM survival in the long-term could be a solution.

IPSDMs did not impact vessel density and contraction duration of the microtissues on day 7, 14 and 21 (Chapter 4, Figure 4B and C). Surprisingly though, previous reports on macrophages suggests their role in supporting vessels and cardiac electrophysiology (Hulsmans et al., 2017; Harari et al., 2017; Hulsmans et al., 2018; Moore et al., 2017; Zaman and Epelman, 2022; Gamrekelashvili et al., 2016; Krishnasamy et al., 2017). However, the parameters that were examined in this study were limited mainly due to the assays and software available. Therefore, these results might not cover all mechanisms that were involved in the cellular crosstalk as we did in the present system, which might explain the lack of confirmation of previous studies. Further investigation on vessel parameters such as diameter or integrity and contraction force and electrophysiological parameters might reveal IPSDM crosstalk with other cell types in the model.
Finally, we collected medium from microtissues (1) without IPSDMs (CMECF); (2) CMECFM0 without M-CSF supplement; (3) CMECFM0 with M-CSF supplement on day 7, 14 and 21. Initially, we determined the secreted cytokine composition in these collected media using multiplexed cytokine assay. However, the cytokine concentrations in CMECFM0 were very close to the upper detection limit of the assay. Because the values were close to the level noise within the assay, it was difficult to draw reliable conclusions. To solve this problem, we concentrated our samples using a column concentrator. Even after this additional step, however, we were only able to detect 4 cytokines, 2 of which were still very low. Cytokine profiles of the CMECFM0, however, showed a mixture of pro- (IL-6 and IP-10) and anti- (IL-10 and IL-1RA) inflammatory cytokines. Although IL-6 and IL-10 concentrations were

very low and therefore might not report accurate values (Chapter 4, Figure 4D, F), the mixed nature of the cytokine profile detected suggested the presence of heterogenous populations of IPSDMs in CMECFM0s. This is similar to the situation *in vivo* (Bajpai et al., 2018). In addition, IL-1RA concentrations was higher than IP-10 concentrations (Chapter 4, Figure 4E, G) which indicates that a majority of IPSDM population in CMECFM0s acquired M2-like anti-inflammatory phenotype while a minor population apparently acquired the M1-like pro-inflammatory phenotype. Unexpectedly, there was a significant increase in all cytokines in CMECFM0 with M-CSF supplement compared to without M-CSF on day 21. This was surprising as the IPSDM numbers on day 21 in these 2 conditions are similar. However, these results might suggest a possible role of M-CSF in triggering a phenotype in IPSDMs in the long-term. More specific assays such as flow cytometry, single cell level assays or staining might be necessary to investigate the effect of microenvironment on IPSDM phenotype.

## 3D Cardiac Models that Integrate Blood Vessel Cells and form functional vasculature

Many of the current 3D cardiac models lack functional vasculature. ECs are key players in the vasculature as they form the inner barrier of vessels and transfer nutrients, oxygen and cells to other tissue components (Aird, 2007, 2012).

Although most of the tissue models previously discussed incorporated primary- or hPSC-derived ECs which self-organized into microvascular-like networks, the models are generally cultured in static conditions, e.g in multiwell plates without fluidic flow. A previous study reported the importance of perfusion for the stability and integrity of vasculature (Ryan et al., 2021). Although microvascular networks inside these 3D models might be lumenized, the lack of fluid flow inside the networks mean that these lumens might collapse through the culture period. Therefore, these microvascular- like networks are not perfusable, and thus in this respect are not functional. In **chapter 5**, we developed an hiPSC-derived perfusable vascularized 3D cardiac microtissues (VMToC) using a commercially available organ-on-chip platform from AimBiotech. We combined 3D cardiac microtissues with single hiPSC-ECs and mural cells in a microfluidic chip under gravity-driven flow. For mural cell component, we used human brain vascular pericytes (HBVPs) as they were commercially available and easy to culture without the extra time and resources needed to differentiate hiPSC. However, hiPSC-derived smooth muscle cells could be used as an alternative (Vila Cuenca et al., 2021).

Initially, we tried different hydrogels such as bovine collagen 1 and Matrigel mix (results not shown in this thesis) as they provide more physiological microenvironment. However, in these mixed hydrogels, cells did not form 3D vessel-like structures, but rather stayed in monolayer. On the other hand, cultures in fibrin hydrogel showed evidence of 3D vessel formation, which was in line with a previous study (Vila Cuenca et al., 2021). Using fibrin hydrogel additionally eliminated batch-to-batch variability of collagen-based hydrogels.

In addition, other critical parameters such as (1) cell numbers to form external vascular networks; and (2) optimal day to integrate microtissues in the chips after seeding of cells to form vasculature, had to be optimized to increase the success and efficiency of the protocol. We found that the higher cell numbers are necessary to fix the formation of the external network and its interconnection/anastomosis with the preexisting internal microvasculature in the microtissues. Notably, for optimal anastomosis, prevascularized microtissues were integrated in the chips as early as day 12 of their static culture period to give enough time to hiPSC-ECs to start the self-organization. Microtissues from later time points were not successfully vascularized, most probably due to the regression of formed microvasculature in the microtissues which hindered anastomosis and perfusion in the chips. After achieving optimal conditions to integrate prevascularized microtissues with hiPSC-ECs and HBVPs in fibrin hydrogel in the chips, we characterized several structural and functional parameters in the chips.

As early as day 1 after seeding, hiPSC-ECs and HBVPs started self-organizing into a complex external vascular network which already showed evidence of anastomosis with the preexisting microvascular network in the cardiac microtissues (Chapter 5, Figure 1). Vascular density was not negatively affected by the presence of contractile cardiac microtissues. Vascular structures in- and around the microtissues were lumenized. That the vessels were lumenized, could be demonstrated by perfusion of fluorescently-tagged beads which followed the whole contour of microvascular networks inside the microtissues (Chapter5, Figure 2). The movement of the beads was oscillatory, following the rhythmic contraction of the cardiac microtissue. This offers the possibility to perfuse immune cells through these vascular networks in the future although it was not done in the present work. It would also address the challenge of incorporation of immune cells into 3D tissues described earlier. We finally showed that anastomosis is fluid flow dependent; continuous perfusion increased both the microvascular and external vascular density (Chapter 5, Figure 3 and 4).

In **chapter 6**, further characterization of contractile dynamics in spontaneous or paced contraction of VMToCs showed significant increase in contraction duration and peak-to-peak time parameters, compared to chip cultures that include only prevascularized cardiac microtissues without the external network around (MToC) (Chapter 6, Figure 1 and 2). This is in-line with a previous study (King et al., 2022), indicating possible crosstalk between EC and CMs. We challenged this crosstalk in two ways: (1) using an inhibitor of nitric oxide synthase (L-NAME) for a short- and long-term; (2) using a pro-inflammatory cytokine (IL-1β) (Chapter 6, Figure 3 and 4). Short-term effects of L-NAME were variable between different hiPSC-CM lines. However, long-term incubation of L-NAME (6h) and IL-1β (12h) showed significant decrease in contraction duration and peak-to-peak time parameters in VMToCs. There was no significant change in these parameters in MToCs. These results indicated increased crosstalk between EC-CM in VMToCs via paracrine signalling, most probably due to the higher numbers of ECs in the culture system. Similar to previous findings (Voges et al., 2023), these results suggest the importance of vascular cells in cardiac functionality and response to drugs.

In **chapter 7**, we described a detailed protocol to generate and characterize VMToCs. With this protocol, it is possible to generate vascularized organoid/microtissues in 5-7 days (Chapter 7, Figure 1-3) efficiently. The protocol described characterization of vessel structures and integrity by bead perfusion assay (Chapter 7, Figure 4). To investigate contractile function, VMToCs can be electrically paced to ensure all microtissues beat at the same rate for measurement (Chapter 7, Figure 5). Functional assays can be carried out as early as day 4, depending on the vascular development. VMToCs can be characterized structurally by fixing and immunofluorescence staining (Chapter 7, Figure 6), although using fluorescently-tagged lines would eliminate an extra staining step besides providing opportunities to live-image tissues and improve image quality.

Compared to the previous 3D cardiac models, the models optimized and developed in this thesis present many advantages, which are summarized as follows;

1. The models can all be based entirely on hiPSC-derived cells. This means that all cellular components can have the same genetic background, important for immune cell interaction but also reducing variability between experiments. In Chapter 5, 6 and 7, primary HBVP were used for convenience but hiPSC-derived smooth muscle cells can replace HBVPs in chips as they are comparable in functional support of ECs (Vila Cuenca et al., 2021).
2. The cardiac microtissues used in this thesis are based on Giacomelli et al., 2020; CMs in microtissues are structurally and functionally similar to neonatal CMs.
3. IPSDMs were successfully integrated into 3D cardiac microtissues, increasing the complexity of the model and providing a basis to study how IPSDMs impact the heart.
4. It is possible to seed chips with fewer cells than used previously in vascularized cardiac tissue models (King et al., 2022).
5. VMToCs provide an *in vitro* perfusable platform to study EC-CM crosstalk without the need for *in vivo* transplantation facilitating the study of EC dysfunction and testing drug responses.

## Limitations and Future perspectives

Although the models described in this thesis could be useful in studying cellular crosstalk in- and with blood vessels, as well as modelling disease and testing drugs, they do still have a number of challenges that remain to be addressed to increase utility. Some of those related as such to the models developed here, are mentioned in the relevant sections above. More general limitations and their impact on possible future perspectives are as follows;

1. Although the models integrate multiple cell types that are important for structure and function, the inclusion of more cell types might lead to more comprehensive modelling of the human heart. For example, the nervous system is important in controlling heart function and addition of sympathetic/parasympathetic or other nerve cells could be useful. Previous studies highlighted the importance of neuronal control on cardiac function such as heart rate and blood flow (Zipes et al., 2017). Dysregulation in heart-

brain axis might cause cardiac pathophysiology (Wake and Brack, 2016). To date, innervation remains largely unexplored in *in vitro* models (Das et al., 2020; Soucy et al., 2020).

2. Incorporation of organoids representing other tissues could extend the utility of the vascularized organoids-on chip models. Other platform examples include vascularization of kidney (Homan et al., 2019), liver (Jin et al., 2018), brain (Salmon et al., 2022), lung (Park et al., 2019; Jain et al., 2018) and different tumor organoids (Sobrino et al., 2016; Straehla et al., 2022). These models are promising to study the impact of fluid flow and increase access to cells and factors in blood/culture medium.
3. Long-term culture might be beneficial in cases where a disease phenotype only emerges late after disease onset, is chronic or long-term or repeated drug exposure is of interest. Long- term culture and the effects on cells in the chips were not investigated. This could also reveal the roles of different cell types in the tissue microenvironment, as in, for example, the IPSDM phenotype described in chapter 4, and CM function described in chapter 5-6.
4. For contractile tissue, contraction force is an important measure of phenotype; it can be affected by many factors including mechanical and chemical stimuli and disease. The analysis software used in this thesis, MuscleMotion, is convenient and low tech, but it gives the contraction amplitude as arbitrary units (Sala et al., 2018). The software was designed to measure relative (pixel)changes in a movie but it is sensitive to light changes. It is therefore not suitable as such to compare contraction amplitude changes between different samples unless light intensities can be carefully controlled. Further investigation for force changes, where for example, cardiac tissue contract against a resistance is a quantitative alternative. Vector flow analysis (Hayakawa et al., 2012) can also provide quantitative readouts but analysis is slow, requiring substantial computer time.
5. Batch-to-batch and even well-to-well variability remains a challenge in 3D tissue cultures. In order to reduce variability arising from other factors besides cell source and culture platform, the on-chip culture conditions should be standardized. Currently, there are many chip designs available commercially or custom-made. However, commercial platforms cannot be easily modified to adapt to the research question. Therefore, many labs prefer custom-made platforms for the ability to modify the design relevant to the questions to be addressed. Due to high costs of commercial platforms and more time-consuming nature of custom-made platforms, currently standardization remains a big challenge, not least in the organ-on-chip field. However, initiatives such as EUROoCS, CEN and CENELEC actively promote and encourage labs to consider more on the standardization practices. Currently, efforts are underway to advance towards this step.
6. Whilst the models described in this thesis may have value in disease modeling (Orlova et al., 2022), for testing pipelines in drug development, higher-throughput platforms would be needed. This is not straightforward with the methods developed here; in addition to standardization, miniaturization and robotics to reduce costs will likely be required.

In the long-term, hiPSC-based 3D cardiac models may in some cases be able to compliment animal models, and provide better outcomes in predicting efficacy and toxicity for humans. As these models carry the genetic background of the individual that they are derived from, they are highly relevant to patients and could be used in precision medicine approaches. However, at present, the models developed in this thesis and described above could still be improved, as indicated. Nevertheless, as they are, they might be able to help bridge the gap between preclinical and clinical research.

## References

Aird, W. C. 2012. *Endothelial cell heterogeneity.* Cold Spring Harbor Perspectives in Medicine 2(1):a006429.

Aird, W. C. 2007. *Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms.* Circulation Research 100(2):158-73.

Bajpai, G., Schneider, C., Wong, N., Bredemeyer, A., Hulsmans, M., Nahrendorf, M., Epelman, S., Kreisel, D., Liu, Y., Itoh, A., et al. 2018. The human heart contains distinct macrophage subsets with divergent origins and functions. Nature Medicine 24:1234–1245.

Beauchamp, P., Jackson, C. B., Ozhathil, L. C., Agarkova, I., Galindo, C. L., Sawyer, D. B., Suter, T. M., and Zuppinger, C. 2020. *3D Co-culture of hiPSC-Derived Cardiomyocytes With Cardiac Fibroblasts Improves Tissue-Like Features of Cardiac Spheroids.* Frontiers in Molecular Biosciences 7:14.

Campostrini, G., Meraviglia, V., Giacomelli, E., van Helden, R. W. J., Yiangou, L., Davis, R. P., Bellin, M., Orlova, V. V., and Mummery, C. L. 2021. *Generation, functional analysis and applications of isogenic three-dimensional self-aggregating cardiac microtissues from human pluripotent stem cells.* Nature Protocols 16 16(4):2213-2256.

Cao, X., van den Hil, F. E., Mummery, C. L., and Orlova, V. V. 2020. G*eneration and Functional Characterization of Monocytes and Macrophages Derived from Human Induced Pluripotent Stem Cells.* Current Protocols in Stem Cell Biology 52(1):e108.

Cao, X., Yakala, G. K., van den Hil, F. E., Cochrane, A., Mummery, C. L., and Orlova, V. V. 2019. *Differentiation and Functional Comparison of Monocytes and Macrophages from hiPSCs with Peripheral Blood Derivatives.* Stem Cell Reports 12(6):1282-1297.

Das, S., Gordián-Vélez, W. J., Ledebur, H. C., Mourkioti, F., Rompolas, P., Chen, H. I., Serruya, M. D., and Cullen, D. K. 2020. *Innervation: the missing link for biofabricated tissues and organs.* npj Regenerative Medicine 5,11.

Dick, S. A., Macklin, J. A., Nejat, S., Momen, A., Clemente-Casares, X., Althagafi, M. G., Chen, J., Kantores, C., Hosseinzadeh, S., Aronoff, L., et al. 2019. *Self-renewing resident cardiac macrophages limit adverse remodeling following myocardial infarction.* Nature Immunology 20 20(1):29-39.

Epelman, S., Lavine, K. J., Beaudin, A. E., Sojka, D. K., Carrero, J. A., Calderon, B., Brija, T., Gautier, E. L., Ivanov, S., Satpathy, A. T., et al. 2014. *Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during inflammation.* Immunity 40(1):91-104.

Gamrekelashvili, J., Giagnorio, R., Jussofie, J., Soehnlein, O., Duchene, J., Briseño, C. G., Ramasamy, S. K., Krishnasamy, K., Limbourg, A., Kapanadze, T., et al. 2016. *Regulation of monocyte cell fate by blood vessels mediated by Notch signalling.* Nature Communications 7:12597.

Giacomelli, E., Bellin, M., Orlova, V. V, and Mummery, C. L. 2017a. *Co-Differentiation of Human Pluripotent Stem Cells-Derived Cardiomyocytes and Endothelial Cells from Cardiac Mesoderm Provides a Three-Dimensional Model of Cardiac Microtissue.* Current protocols in human genetics 95:21.9.1-21.9.22.

Giacomelli, E., Bellin, M., Sala, L., Meer, B. J. van, Tertoolen, L. G. J., Orlova, V. V, and Mummery, C. L. 2017b. *Three-dimensional cardiac microtissues composed of cardiomyocytes and endothelial cells co-differentiated from human pluripotent stem cells.* Development 144:1008–1017.

Giacomelli, E., Meraviglia, V., Campostrini, G., Cochrane, A., Cao, X., van Helden, R. W. J., Krotenberg Garcia, A., Mircea, M., Kostidis, S., Davis, R. P., et al. 2020. *Human-iPSC-Derived Cardiac Stromal Cells Enhance Maturation in 3D Cardiac Microtissues and Reveal Non-cardiomyocyte Contributions to Heart Disease.* Cell Stem Cell 26:862-879.e11.

Harari, E., Guo, L., Smith, S. L., Braumann, R. E., Virmani, R., and Finn, A. V. 2017. H*eart-resident macrophages: Are they involved in the rhythm of every beat?* Journal of Thoracic Disease 9(8):2264-2267.

Hayakawa, T., Kunihiro, T., Dowaki, S., Uno, H., Matsui, E., Uchida, M., Kobayashi, S., Yasuda, A., Shimizu, T., and Okano, T. 2012. *Noninvasive evaluation of contractile behavior of cardiomyocyte monolayers based on motion vector analysis.* Tissue Engineering - Part C: Methods 18(1):21-32.

Homan, K. A., Gupta, N., Kroll, K. T., Kolesky, D. B., Skylar-Scott, M., Miyoshi, T., Mau, D., Valerius, M. T., Ferrante, T., Bonventre, J. V., et al. 2019. *Flow-enhanced vascularization and maturation of kidney organoids in vitro.* Nature Methods 16(3):255-262.

Hulsmans, M., Clauss, S., Xiao, L., Aguirre, A. D., King, K. R., Hanley, A., Hucker, W. J., Wülfers, E. M., Seemann, G., Courties, G., et al. 2017. *Macrophages Facilitate Electrical Conduction in the Heart.* Cell 169(3):510-522.e20.

Hulsmans, M., Sager, H. B., Roh, J. D., Valero-Muñoz, M., Houstis, N. E., Iwamoto, Y., Sun, Y., Wilson, R. M., Wojtkiewicz, G., Tricot, B., et al. 2018. *Cardiac macrophages promote diastolic dysfunction.* Journal of Experimental Medicine 215 (2): 423–440.

Jain, A., Barrile, R., van der Meer, A. D., Mammoto, A., Mammoto, T., De Ceunynck, K., Aisiku, O., Otieno, M. A., Louden, C. S., Hamilton, G. A., et al. 2018. *Primary Human Lung Alveolus-on-a- chip Model of Intravascular Thrombosis for Assessment of Therapeutics.* Clinical Pharmacology and Therapeutics 103(2):332-340.

Jin, Y., Kim, J., Lee, J. S., Min, S., Kim, S., Ahn, D. H., Kim, Y. G., and Cho, S. W. 2018. *Vascularized Liver Organoids Generated Using Induced Hepatic Tissue and Dynamic Liver-Specific Microenvironment as a Drug Testing Platform.* Advanced Functional Materials 28,37.

King, O., Cruz-Moreira, D., Sayed, A., Stevens, M. M., Rasponi, M., and Terracciano, C. M. 2022. *Functional microvascularization of human myocardium in vitro.* Cell Reports Methods:100280.

Krishnasamy, K., Limbourg, A., Kapanadze, T., Gamrekelashvili, J., Beger, C., Häger, C., Lozanovski, V. J., Falk, C. S., Napp, L. C., Bauersachs, J., et al. 2017. *Blood vessel control of macrophage maturation promotes arteriogenesis in ischemia.* Nature Communications 8(1):952.

Loh, K. M. M., Chen, A., Koh, P. W. W., Deng, T. Z. Z., Sinha, R., Tsai, J. M. M., Barkal, A. A. A., Shen, K. Y. Y., Jain, R., Morganti, R. M. M., et al. 2016. *Mapping the Pairwise Choices Leading from Pluripotency to Human Bone, Heart, and Other Mesoderm Cell Types.* Cell 166(2):451-467.

Mills, R. J., Parker, B. L., Quaife-Ryan, G. A., Voges, H. K., Needham, E. J., Bornot, A., Ding, M., Andersson, H., Polla, M., Elliott, D. A., et al. 2019. *Drug Screening in Human PSC-Cardiac Organoids Identifies Pro-proliferative Compounds Acting via the Mevalonate Pathway.* Cell Stem Cell 24:895-907.e6.

Moore, E. M., Ying, G., and West, J. L. 2017. *Macrophages Influence Vessel Formation in 3D Bioactive Hydrogels.* Advanced Biosystems 1.

Orlova, V. V., Van Den Hil, F. E., Petrus-Reurer, S., Drabsch, Y., Ten Dijke, P., and Mummery, C. L. 2014. *Generation, expansion and functional analysis of endothelial cells and pericytes derived from human pluripotent stem cells.* Nature Protocols 9(6):1514-31.

Orlova, V. V., Nahon, D. M., Cochrane, A., Cao, X., Freund, C., van den Hil, F., Westermann, C. J. J., Snijder, R. J., Ploos van Amstel, J. K., ten Dijke, P., et al. 2022. *Vascular defects associated with hereditary hemorrhagic telangiectasia revealed in patient-derived isogenic iPSCs in 3D vessels on chip.* Stem Cell Reports 17(7):1536-1545.

Otero, K., Turnbull, I. R., Poliani, P. L., Vermi, W., Cerutti, E., Aoshi, T., Tassi, I., Takai, T., Stanley, S. L., Miller, M., et al. 2009. *Macrophage colony-stimulating factor induces the proliferation and survival of macrophages via a pathway involving DAP12 and β-catenin.* Nature Immunology 10(7):734-43.

Park, J. Y., Ryu, H., Lee, B., Ha, D. H., Ahn, M., Kim, S., Kim, J. Y., Jeon, N. L., and Cho, D. W. 2019. *Development of a functional airway-on-a-chip by 3D cell printing.* Biofabrication 11(1):015002.

Richards, D. J., Li, Y., Kerr, C. M., Yao, J., Beeson, G. C., Coyle, R. C., Chen, X., Jia, J., Damon, B., Wilson, R., et al. 2020. *Human cardiac organoids for the modelling of myocardial infarction and drug cardiotoxicity.* Nature biomedical engineering 4:446–462.

Ryan, A. R., England, A. R., Chaney, C. P., Cowdin, M. A., Hiltabidle, M., Daniel, E., Gupta, A. K., Oxburgh, L., Carroll, T. J., and Cleaver, O. 2021. *Vascular deficiencies in renal organoids and ex vivo kidney organogenesis.* Developmental Biology 477:98-116.

Sala, L., Van Meer, B. J., Tertoolen, L. G. J., Bakkers, J., Bellin, M., Davis, R. P., Denning, C., Dieben, M. A. E., Eschenhagen, T., Giacomelli, E., et al. 2018. *Musclemotion: A versatile open software tool to quantify cardiomyocyte and cardiac muscle contraction in vitro and in vivo.* Circulation Research 122(3):e5-e16..

Salmon, I., Grebenyuk, S., Abdel Fattah, A. R., Rustandi, G., Pilkington, T., Verfaillie, C., and Ranga, A. 2022. *Engineering neurovascular organoids with 3D printed microfluidic chips.* Lab on a Chip 22, 1615-1629.

Sobrino, A., Phan, D. T. T., Datta, R., Wang, X., Hachey, S. J., Romero-López, M., Gratton, E., Lee, A. P., George, S. C., and Hughes, C. C. W. 2016. *3D microtumors in vitro supported by perfused vascular networks.* Scientific Reports 6:31589.

Soucy, J. R., Bindas, A. J., Brady, R., Torregrosa, T., Denoncourt, C. M., Hosic, S., Dai, G., Koppes, A. N., and Koppes, R. A. 2020. *Reconfigurable Microphysiological Systems for Modeling Innervation and Multitissue Interactions.* Advanced Biosystems 4(9):e2000133.

Straehla, J. P., Hajal, C., Safford, H. C., Offeddu, G. S., Boehnke, N., Dacoba, T. G., Wyckoff, J., Kamm, R. D., and Hammond, P. T. 2022. *A predictive microfluidic model of human glioblastoma to assess trafficking of blood–brain barrier-penetrant nanoparticles.* Proceedings of the National Academy of Sciences of the United States of America 119(23):e2118697119.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. 2007. *Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors.* Cell 131(5):861-72.

Takahashi, K., Yamanaka, S., Zhang, Y., Li, Y., Feng, C., Li, X., Lin, L., Guo, L., Wang, H., Liu, C., et al. 2006. *Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors.* Cell 126(4):663-76.

Thomson, J. A. 1998. Embryonic stem cell lines derived from human blastocysts. Science 282(5391):1145-7.

Vila Cuenca, M., Cochrane, A., van den Hil, F. E., de Vries, A. A. F., Lesnik Oberstein, S. A. J., Mummery, C. L., and Orlova, V. V. 2021. *Engineered 3D vessel-on-chip using hiPSC-derived endothelial- and vascular smooth muscle cells.* Stem Cell Reports 16(9):2159-2168.

Voges, H. K., Foster, S. R., Reynolds, L., Parker, B. L., Devilée, L., Quaife-Ryan, G. A., Fortuna, P. R. J., Mathieson, E., Fitzsimmons, R., Lor, M., et al. 2023. *Vascular cells improve functionality of human cardiac organoids.* Cell Reports 42(5):112322.

Wake, E., and Brack, K. 2016. Characterization of the intrinsic cardiac nervous system. Autonomic neuroscience : basic & clinical 199:3–16.

Zaman, R., and Epelman, S. 2022. *Resident cardiac macrophages: Heterogeneity and function in health and disease.* Immunity 55(9):1549-1563.

Zipes, D. P., Jalife, J., and Stevenson, W. G. 2017. *Cardiac Electrophysiology: From Cell to Bedside: Seventh Edition.*