Research Strategy

Phase I Progress Report

Phase I Highlights

- **Enhanced & Rapid hiPSC-CM Structural Maturation**. Building upon preliminary reports[1], we published a paper in *Scientific Reports*[2] showing enhanced hiPSC-CM maturation using CARTOX's MATURA96 plating approach. Enhanced hiPSC-CM structural maturation was determined by probing for mature markers: hypertrophy, reduced circularity, β-myosin expression, myosin light chain 2v (ventricular) expression, SERCA2 expression and multi-nucleation. Figure 1 shows some of the highlights from our *Scientific Reports* manuscript showing hiPSC-CM structural maturation. Maturation requires one week after plating hiPSC-CMs. Using CARTOX plating approaches it is possible to dictate the maturation state, which enables cardiotoxicity screening using fetal and/or mature hiPSC-CMs which adds value to drug discovery projects.

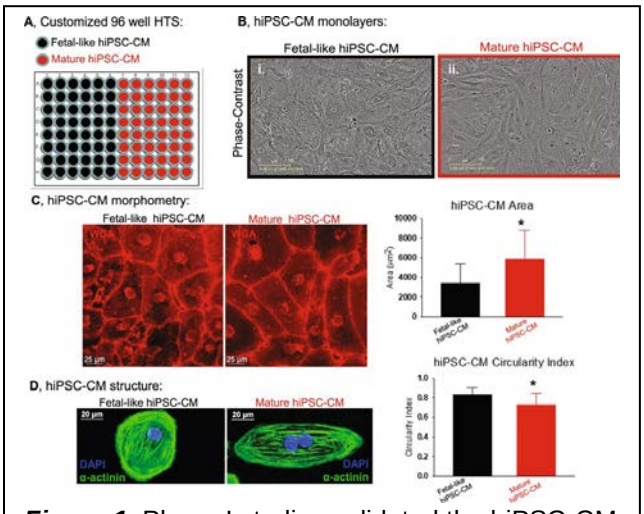


Figure 1. Phase I studies validated the hiPSC-CM maturation process using MATURA96 (*A*). *B*, Phase contrast images of hiPSC-CM monolayers. *C*, WGA-staining cell membranes shows hypertrophy and rectangular shape of mature hiPSC-CMs. *D*, Fluorescent images of single hiPSC-CMs.

- **Enhanced & Rapid hiPSC-CM Functional Maturation.** CARTOX plating approaches induce expression of Kir2.1, the potassium channel that plays a major role in cardiac repolarization and setting the resting membrane potential.[1] In phase I we used the specific hERG channel blocker, E-4031, to demonstrate significant maturation of hiPSC-CMs, and less dependence of mature hiPSC-CMs on the hERG ion channel for repolarization (figure 2). Similar results were obtained using other hERG blockers like dofetilide. This is in contrast to previous reports using fetal hiPSC-CMs showing that the hERG channel exclusively sets the resting membrane potential of immature hiPSC-CM cells.[3]

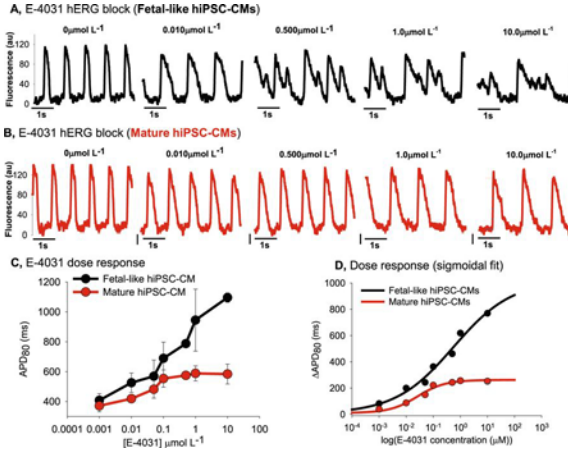


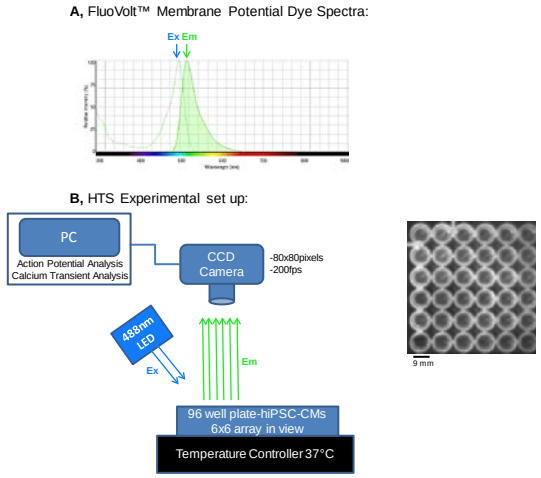
Figure 2. Phase I studies show mature hiPSC-CM monolayers are less sensitive to hERG ion channel blockade.

- **hiPSC-CM Maturation Determines Drug Responsiveness.** One of the most exciting highlights from Phase I studies was the discovery that hiPSC-CM maturation state determines responsiveness to many drugs. For example, fluoxetine (Prozac) completely arrested immature fetal like hiPSC-CMs, while mature hiPSC-CMs survived the same concentration of drug (10μM with no harmful side effects).
- **Matura96/24 Electrophysiology Screen Consistency and Predictivity.** Phase I SBIR studies utilized MATURA 96 well plates for parameters of spontaneous beat rate and action potential duration, coefficients of variance were shown to be below 11% well to well or 10% plate to plate. TdP risk prediction analysis produced very good AUC values from ROC curves for high and intermediate risk CiPA drugs screened: AUC=0.8542.
- **Streamlined PDMS bottom plate production.** Plate production did rely on manually inserting pre cut PDMS coverslips in to each well of a multi well plate-even 96 well plates. In Phase I we improved this process by using bottomless plates and adhering PDMS sheets to form the plate bottom. This improvement in maturation plate production saves time and resources significantly.
- **Natural ECM derived from human stem cells (collaboration with StemBioSys).** An exciting collaborative partnership with StemBioSys was initiated toward the end of phase I studies. Preliminary data

indicates improved cardiotoxicity screening assays using StemBioSys' advanced extracellular matrix produced by human cells to replicate a 3-dimensional microenvironment "home" for hiPSC-CMs.

- **Conclusion and Future Directions.** Phase I studies enabled CARTOX to validate its core MATURA96 hiPSC-CM plating approach and showed its utility for high throughput cardiotoxicity and pro-arrhythmia screening. In Phase II studies we will develop MATURA384, a commercial scale optical mapping set up for final consumers using off the shelf components and further validate the novel human cell ECM generated by StemBioSys. Completion of this Phase II SBIR project will position CARTOX for commercialization of its technology and further advance the use of hiPSC-CM assays for drug discovery and toxicity screening. Towards this end, CARTOX has begun license negotiations with Canopy Bioscience and StemBioSys for marketing and distribution of MATURA96 plates and mature hiPSC-CMs (see attached letter of support).

Figure 3. High throughput hiPSC-CM optical mapping using MATURA96.

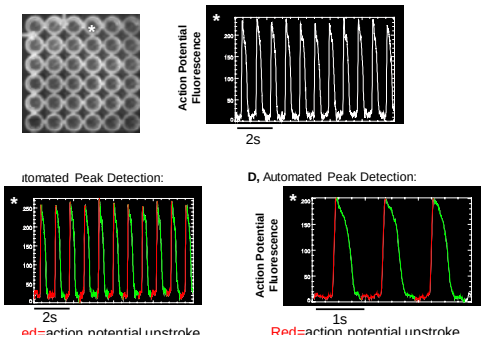


Phase I Results Specific Aim 1: Validate the MATURA96/24 human electrophysiology platforms for predictive acute cardiotoxicity and proarrhythmia screening.

1) Optical mapping prototype currently in use: We validated the MATURA96 hiPSC-CM platform using a voltage sensitive dye (FluoVolt™, ThermoFisher) and high speed CCD camera (SciMeasure, 200fps). Details of the optical mapping set up have been described before.[4] The current optical mapping platform is depicted in figure 3. We have found that FluoVolt™ is the optimal voltage sensitive dye for these experiments owing to its low cytotoxicity profile and high fluorescence emission upon membrane depolarization.[5] An advantage of this optical mapping assay is that it can accommodate cell culture vessels of nearly any size and multi-well format (6-, 12-, 24-, 48-, 96-well plates). In phase I we utilized 96 and 24 well plates. Figure 3 shows that currently we image a 6x6 array of a 96 well plate in one recording. Mechanical translation of each plate under the camera enables interrogation of the entire 96 wells in just over 1 min of imaging time. In phase II experiments we will develop an advanced optical mapping assay which is capable of imaging the entire 96 well plate in one recording.

In phase I CARTOX also developed automated peak detection software for action potential or calcium transient analysis. Figure 4 shows an example of the automated peak detection for a MATURA96 plate. This enables robust, unbiased detection of action potential depolarization and repolarization. In this analysis the upstroke of the action potential is colored in red and the repolarization phase is colored green.

Figure 4. Automated peak detection algorithms have been developed for EP analysis of cardiac depolarization and repolarization phases of the action potential.



2) Screening CiPA Compounds: We have validated MATURA96 using 12 CiPA compounds as part of the myocyte validation study. Our laboratory participated as a non-core site in the phase II cardiomyocyte validation efforts. This validation was done in a blinded way with the users/operator/data analysis blind to the identity of each compound. The high throughput nature of MATURA96 enabled us to perform this validation screening using three unique hiPSC-CM cell types: two commercially available cell lines (**CDI**=Cellular Dynamic International iCell[2] & **CLS**=Cellartis, Takara Bio) and one in house generated hiPSC-CM cell line (**MCH**=Michigan). These cell types each differ in their patient-genetic origin and in the case of the **CLS** cardiomyocytes, purity. The **CLS** cardiomyocytes are not purified

and contain ~25% non-cardiomyocytes. On the other hand, **CDI** and **MCH** cells are purified to ~98% pure cTnT+ cells. CDI hiPSC-CMs are genetically purified using genetic modification, while MCH hiPSC-CMs are purified using magnetic activated cell sorting (MACS) approaches developed in collaboration with Miltenyi Biotec. MACS technology is FDA approved for the production of cells used in clinical cell therapies.[6] This difference in purity is manifested as unique cardiac electrophysiology parameters at baseline (figure 5). In particular, the action potential shape is much different in the **CLS** cells with pronounced phase 4 depolarization. Baseline differences were quantified (figure 5) and the **CLS** hiPSC-CMs exhibited slower spontaneous beat rate and significantly longer action potential duration 90 (APD90). The **CDI** and **MCH** cell lines were similar to each other at baseline. Not surprisingly, each cell type responded to drug compounds in slightly different ways.

Figure 5. Screening CiPA compounds using 3 different hiPSC-CM cell types. *denotes significant difference, ANOVA.

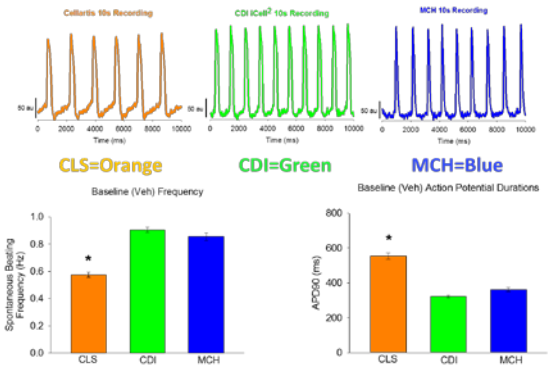


Figure 6. Low Risk CiPA Compounds Tested in MATURA96

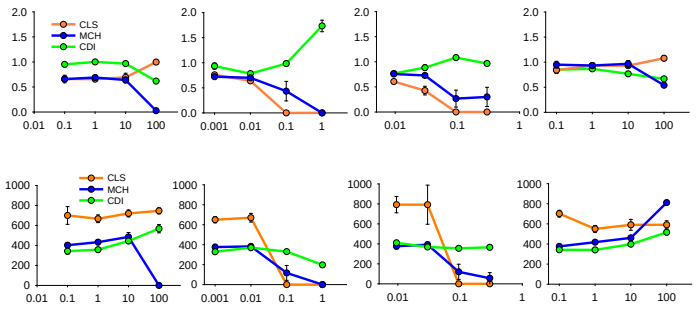


Figure 7. Intermediate Risk CiPA Compounds Tested in MATURA96.

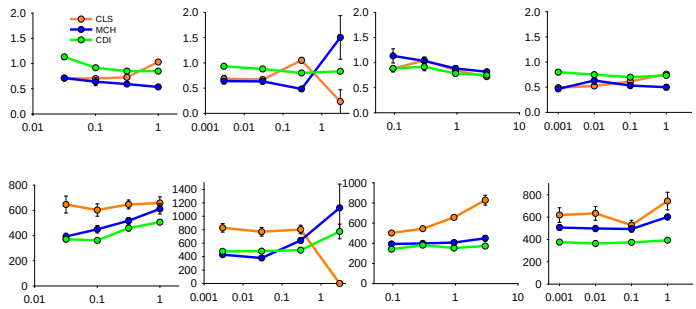
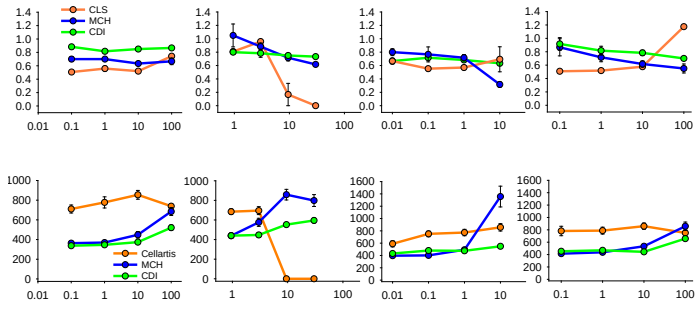


Figure 8. High Risk CiPA compounds tested in MATURA96.



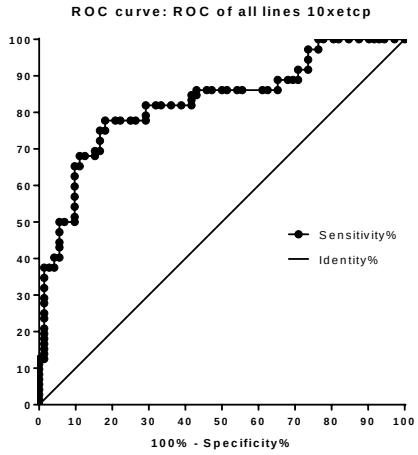
Low Risk Compounds (4). Low risk compounds tested using MATURA96 include mexiletine, nifedipine, nitrendipine and ranolazine (figure 6, A=spontaneous rhythm in Hz; B=APD90 in ms). The concentration of each compound was based on the effective therapeutic plasma concentration (ETPC) from clinical data and was recommended by the CiPA Steering Committee. Each cell type responded a bit differently to each compound. For example, Cellartis (**CLS**) and Michigan (**MCH**) cells were very sensitive to calcium channel blockers (nifedipine & nitrendipine), while **CDI** cells were much less sensitive.

Intermediate Risk Compounds (4). Intermediate risk compounds tested using MATURA96 include droperidol, domperidone, clozapine and terfenadine (figure 7). These compounds caused APD90 prolongation with escalating dose, with sensitivity being different for each hiPSC-CM cell type. Clozapine, for example, showed the greatest effects in the unpurified **CLS** cells, which indicates that the presence of non-cardiomyocytes exacerbates the effect on cardiac repolarization.

High Risk Compounds (4). High risk compounds tested using MATURA96 included Disopyramide, quinidine, vandetanib and D,l Sotalol (figure 8). These compounds caused significantly greater APD90 prolongation compared to the intermediate risk compounds, as expected. Interestingly, quinidine caused hiPSC-CM quiescence only in the **CLS** cells, again pointing to the important issue of cell purity and the potential contribution of non-myocytes to drug responsiveness.

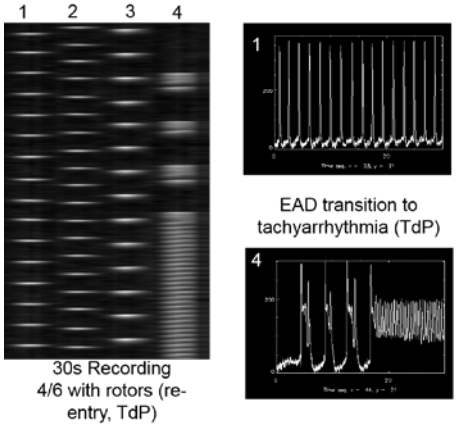
3) MATURA96 receiver operating characteristic (ROC). ROC curves are used to describe the usefulness of tests. We calculated ROC curves for all three of the cell lines tested using MATURA96. Figure 9 shows the ROC curve, with area under the curve (AUC)=0.8542 when analyzing all compounds from high and intermediate risk categories for arrhythmia. This high AUC indicates very good sensitivity and specificity for the MATURA96 assay. *In short, MATURA96 is a robust and reliable pro arrhythmia screening assay with advantages over the current pre-clinical cardiotoxicity testing paradigm.*

Figure 9. ROC curves indicate excellent predictive power of MATURA96 to detect proarrhythmia with multiple cell lines. *AUC=0.8542*



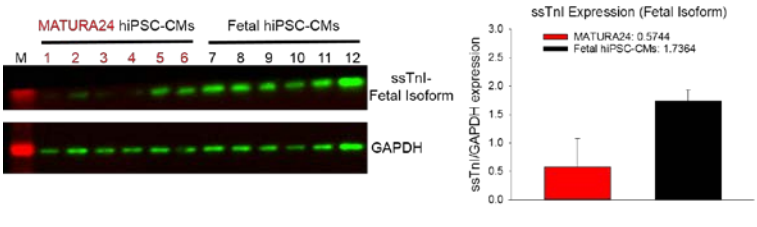
4) TdP in a dish using MATURA96. The high spatial resolution of our optical mapping approach enables the visualization of drug induced TdP in a dish. Domperidone (dopamine D2 receptor antagonist, antiemetic) is a drug prescribed for new mothers to increase lactation, but can also cause fatal arrhythmias in some women.[7] Our blinded validation of MATURA96 included domperidone at concentrations of 0.003, 0.03, 0.3, and 3.0µM. CiPA classified Domperidone as an intermediate risk compound, so it was expected to observe APD prolongation with escalating dose (figure 10). It was surprising to observe electrical rotors, indicative of re-entrant arrhythmia in 67% (4/6 monolayers) of the monolayers given the highest concentration (3.0µM). Figure 10 shows the unique ability of our MATURA96 platform to observe drug induced TdP *in vitro*. We constructed time-space plots shown in figure 10, where time 0 is at the top of the panel (30s total recording) and spontaneous activations are shown for four different concentrations of domperidone (1=0.003 µM; 2=0.03 µM; 3=0.3 µM; 4=3.0 µM). The time space plot is similar to a confocal line scan, commonly used to visualize calcium flux in cardiomyocytes. Concentration 3 shows significant APD prolongation and concentration 4 shows drug induced early after depolarization (EAD) that converted to tachyarrhythmia which is characterized by a stable high frequency rotor. This exciting result suggests that we may be able to move away from reliance on surrogates of arrhythmia (APD prolongation) towards direct visualization of drug induced TdP. *Phase II efforts will further validate this finding using a greater number of CiPA compounds to determine the utility of using TdP as an arrhythmia category for CiPA assays.*

Figure 10. Drug induced TdP in a dish. Domperidone was administered at 4 different concentraitons. TdP was observed at the highest concentration (4). See text for details.



5) Reduction of fetal myofilament isoform expression of troponin (ssTnI). Postnatal, the mammalian heart changes the cardiac myofilament gene expression program. Specifically, the troponin I isoform is well known to change from a fetal isoform (ssTnI) to a mature cardiac isoform (cTnI).[8] In phase I SBIR experiments we determined the expression of the fetal cardiac troponin isoform, slow skeletal troponin I (ssTnI) in MATURA24 plates. Figure 11 indicates reduced protein expression of ssTnI in hiPSC-CMs from CDI plated in MATURA24 plates. ssTnI expression relative to GAPDH is quantified and plotted in the right panel. This indicates a transition away from fetal gene expression towards adult cardiomyocyte gene expression.

Figure 11. Mature hiPSC-CMs have reduced expression of fetal troponin I, ssTnI.



Phase I Results Specific Aim 2: Validate the MATURA96/24 human electrophysiology platforms for predictive chronic cardiotoxicity and proarrhythmia effects of oncology drugs.

Figure 12. MATURA96 hiPSC-CMs have shorter APD than immature hiPSC-CMs at baseline.

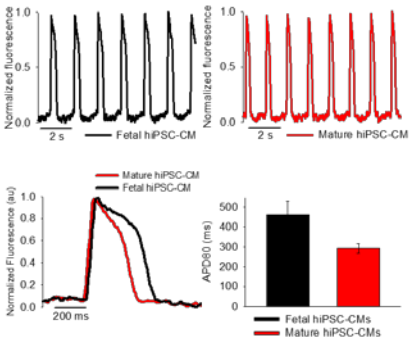
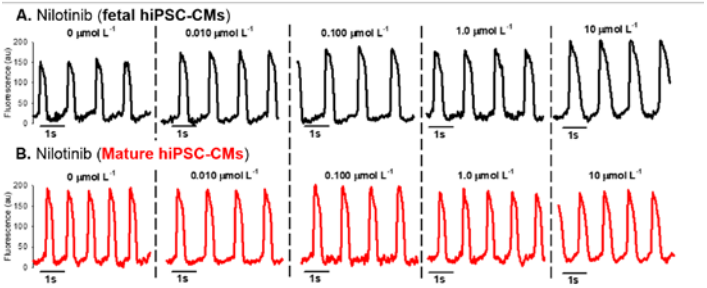


Figure 12 shows that mature hiPSC-CMs have shorter APD at baseline than immature (**fetal**) hiPSC-CMs. This is because mature hiPSC-CMs cultured in the MATURA96 platform express significant amounts of Kir2.1, the potassium channel that sets the resting membrane potential and contributes to the repolarization phase of the cardiac action potential.[1, 2]

1) ProArrhythmia screening of chemotherapy. We *hypothesized* that this difference in APD at baseline translates to a different sensitivity of mature hiPSC-CMs to chemotherapy reagents that carry TdP risk like nilotinib. Nilotinib (Tasigna) is a tyrosine kinase inhibitor used for the treatment of leukemia and is being investigated for treatment of Parkinson's and other neurological disorders. One adverse effect of nilotinib is QT prolongation. In phase I we tested the effect of nilotinib using **immature** and mature hiPSC-CMs in our HTS electrophysiology assay. Figure 13 shows the effect of nilotinib to prolong the action potential duration in a dose dependent manner. We also used the MATURA96 platform to screen for nilotinib effects on the hiPSC-CM monolayer intracellular calcium flux (data not shown, but published[2]). Nilotinib increased intracellular calcium concentrations in a dose dependent manner, with the effect being greatest in the fetal like hiPSC-CM monolayers. This also demonstrated that MATURA96 can be utilized for assessment of multiple cardiac electrophysiology parameters including membrane voltage as well as intracellular calcium flux. Use of VSD technology enables multi parameter assays.

Figure 13. Phase I results for proarrhythmia effects of nilotinib, a chemotherapy compound. Action potential monitoring was done using MATURA96 as well as immature hiPSC-CMs for comparison.



2) Chronic Cardiotoxicity Following Chemotherapy Exposure (Doxorubicin). Figure 14 shows that in Phase I we were able to utilize hiPSC-CMs plated in MATURA96 to monitor chemotherapy induced cardiotoxicity over 13 days. In this label free assay, hiPSC-CMs were plated as highly confluent monolayers and doxorubicin (1µM) was given at time 0. Continuous time-lapse monitoring of the entire 96 well plate, with images acquired every 2h, was used to determine hiPSC-CM monolayer structural health (Incucyte, Essen Bioscience, Ann Arbor, MI). In this assay phase contrast images were collected over the course of the experiment and image analysis was performed to create a confluence mask (shown in goldenrod, figure 14) that can discriminate between the presence or absence of cells. Doxorubicin, a commonly used chemotherapy for many types of cancers and has well known cardiotoxic side effects and this assay can detect this structural cardiotoxicity.[9] At time 0 monolayer confluence is near 100% and this confluence value drops with application of doxorubicin (figure 14, panel D), thus indicating structural cardiotoxicity. We also have utilized AnnexinV reagents to probe doxorubicin induced apoptosis with success using the Incucyte instrument in fluorescence detection mode (data not shown). Quantification of chemotherapy induced cardiotoxicity is in figure 15. Vehicle control (DMSO) delivery to hiPSC-CM monolayers did not result in cardiotoxic cell death and

Figure 14. Long term chemotherapy induced cardiotoxicity monitoring assay. Confluence mask=goldenrod

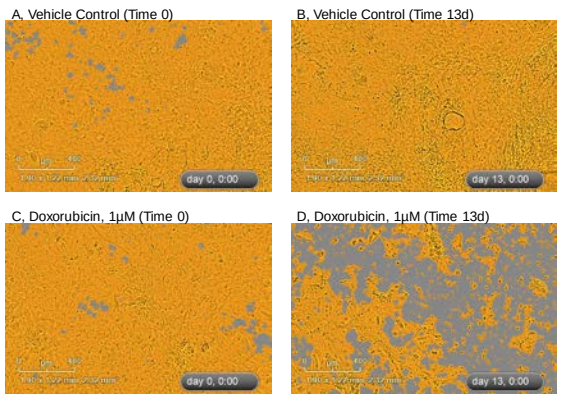
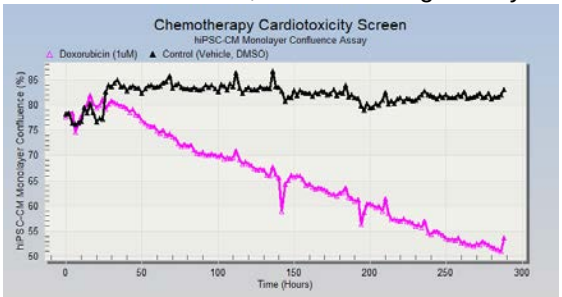


Figure 15. hiPSC-CM label free cardiotoxicity screening. This quantification is made using the images in figure 14. Doxorubicin (pink) significantly reduces hiPSC-CM monolayer confluence over time, thus indicating toxicity.



monolayer structural defects (black symbols). Doxorubicin treatment (pink, 1µM), however resulted in steady decline of hiPSC-CM monolayer confluence, which indicates cardiotoxicity.

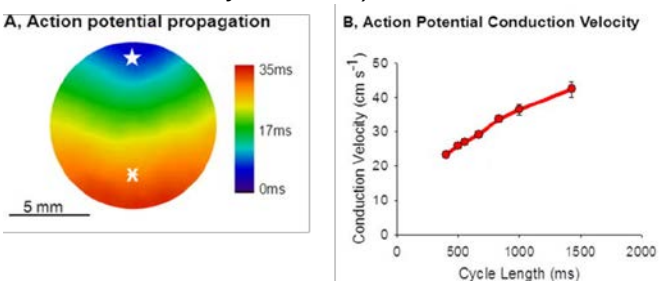
Phase II Significance.

The FDA has been calling for a paradigm change for pre-clinical cardiotoxicity screening requirements.[10, 11] The current International Committee on Harmonization (ICH) recommendations call for the use of genetically modified human embryonic kidney (HEK) cells or CHO cells that overexpress specific ion channels (e.g., hERG channels, hERG assay, calcium channels, etc.) for pre-clinical drug development safety screening. It is now recognized that this reliance on screening a compound's effects on a single ion channel overexpressed in a cell system that does not contract and does not possess the complex complement of ion channels in the cardiomyocyte is not accurate**.**[12, 13] The reliance on the hERG assay as the gate keeper for drug discovery projects has resulted in the premature termination of drug discovery projects, mislabeling of drug warnings and missed opportunities for development of safe and effective drugs.[11] The ideal pre-clinical cardiotoxicity screening assay should include the use of human cardiomyocytes for more predictive value. Thus, development of new and improved hiPSC-CM electrophysiological screening assays (like CARTOX MATURA multi-well assays) is significant to the FDA and major stakeholders in the drug discovery, development and commercialization arenas. Our proposal also helps to address the 3R's of animal use: Replace, Reduce, Refine. Furthermore, adoption and use of hiPSC-CMs is predicted to significantly reduce the cost of the drug discovery and development processes.

The goals of this Phase II SBIR application are significant to advancement of adoption of the Comprehensive *In Vitro* Proarrhythmia Assay (CiPA). CiPA is positioned to augment or replace the hERG assay and possibly the requirement for a thorough QT (TQT) study using animals. One part of CiPA involves testing compound effects using human stem cell derived cardiomyocytes, and validation studies have indicated good sensitivity and specificity of assays using multi-electrode array (MEA) technologies and voltage sensitive dyes (VSD).[14, 15] Currently VSD technology does not have a significant market presence due to the lack of turn-key optical mapping instruments that can be utilized in the laboratories of contract research organizations and Big Pharma. CARTOX hiPSC-CM electrophysiology assays rely on the use of VSD approaches, and in Phase II we will build and validate a final user commercially suitable hiPSC-CM cardiac optical mapping instrument using off-the-shelf components.

Comparison of MEA and VSD approaches. While MEA approaches enable continuous data streaming, are easy to use, and have a strong market presence; MEA recordings have inherent limitations. *First*, MEA recordings do not provide information on cardiac action potential morphology. Rather, MEA recordings report on field potential duration (FPD)-a surrogate marker for the action potential duration (APD). The phase of the action potential that is most affected (early vs. late repolarization) by a compound is not reliably detectable using MEA approaches and thus MEA assays cannot provide clues as to the precise mechanism of action of a compound that has pro arrhythmia risk. *Second*, MEA recordings do not provide high fidelity data on the action potential impulse conduction velocity and pattern. This is a major limitation because reduced conduction velocity of the cardiac action potential predisposes to fatal cardiac arrhythmias.[16-18] The spacing distance between recording electrodes in MEA systems results

Figure 16. Use of VSD approaches enables the quantification of hiPSC-CM action potential impulse propagation pattern (A, activation map) and velocity (B, Conduction Velocity restitution).



in low spatial resolution of monolayer recordings and lack of high fidelity information on electrical wave propagation. VSD approaches enable direct visualization of activation patterns and quantification of velocity (figure 16, adapted from Herron et al.[1]). *Third*, MEA technology requires contact between the cells and each electrode. This feature precludes significant modification of the extracellular matrix between the electrodes and cells. We and others have found that the ECM stiffness is critical for hiPSC-CM maturation[1, 19] and these approaches to mature the hiPSC-CM phenotype are not feasible using MEA assays. 3D engineered heart tissue is emerging as the next technological advance for hiPSC-CM based cardiotoxicity screening[20] and MEA systems are not immediately available to measure from these novel approaches. Our VSD approach overcomes these limitations presented by the MEA recording approaches and completion of this Phase II SBIR project is highly significant to the advancement of CiPA and improved pre-clinical cardiotoxicity and proarrhythmia screening. There is considerable commercial interest from outside parties to license our technology and partner for sales and distribution (see letters of support from CDI, Canopy Biosciences, SBS and investment banker).

In summary, the assays being developed by CARTOX in collaboration with StemBioSys are very significant to the CiPA initiative and to the overall mission of the NIH and FDA.

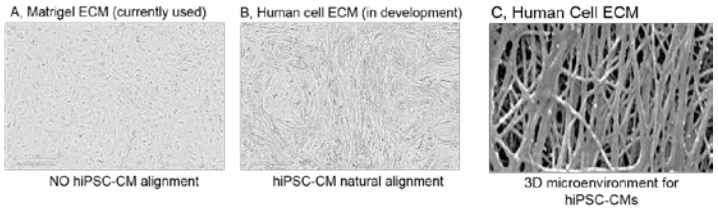
Phase II Innovation.

This proposal will develop innovative hiPSC-CM plating and imaging technologies. The vast majority of currently used hiPSC-CM proarrhythmia screening assays rely on immature, fetal-like cells that do not resemble the structure or function of the adult cardiomyocyte. In Phase I studies we were able to demonstrate the importance of hiPSC-CM maturation state for pro arrhythmia compound screening.[2] CARTOX's innovative mature hiPSC-CM screening assay enables testing using human cardiac cells that express significant amounts of adult cardiomyocyte repolarizing currents (Kir2.1) relative to fetal cells.[1, 2] This difference has major impact on proarrhythmia screening. Also, the vast majority of hiPSC-CM pro arrhythmia screens rely solely on field potential duration (MEA technology) or action potential duration prolongation (VSD technology) as surrogate markers for TdP induction. This is a limitation because not all drugs that prolong the action potential (QT interval) will cause fatal TdP arrhythmias. A key innovative aspect of this phase II proposal is the development and validation of a novel human stem cell derived ECM that in preliminary studies appears to discriminate between safe compounds that prolong the APD and high risk compounds that prolong the APD and lead to TdP.

Preliminary Results in support of Phase II Aims.

1. **Natural Stem Cell Derived ECM.** Figure 17 shows that StemBioSys' natural human stem cell derived ECM promotes complex alignment of hiPSC-CM monolayers. StemBioSys' approach to generating natural human stem cell derived ECM coatings for multi-well plates and cell culture is leading the next evolution in stem cell research. In their ECM product, human stem cells are cultured and then induced to secrete ECM. Subsequently cells are gently washed away and the natural cell generated ECM remains on the cell culture dish and can be used for seeding hiPSC-CMs. This is in contrast to assays that rely on engineering of cell culture plates to force alignment in one direction (e.g., microheart assay). The heart is a complex organ with cellular/fiber alignment and orientation occurring in a non- linear fashion. Figure 17C shows an electron micrograph of StemBioSys' current ECM product, Cellvo™ which produces a natural 3D microenvironment to culture cells on. The natural stem cell derived ECM to be developed here for inclusion in the MATURA96 hiPSC-CM EP screening assay is a distinct process (but similar) from the

Figure 17. StemBioSys' proprietary natural human cell derived ECM promotes alignment of hiPSC-CMs (B) compared to currently used Matrigel ECM (A). C, electron micrograph of StemBioSys' natural ECM shows 3D microenvironment for hiPSC-CM cell culture and maintenance.



Cellvo™ product, and together with StemBioSys we are submitting invention disclosures and generating a patent application to protect this intellectual property.

2. **Natural Stem Cell Derived ECM promotes the occurrence of drug induced TdP in a Dish.** We have found a novel approach for discriminating between drugs that prolong the action potential duration, but do not cause arrhythmias (low risk) and drugs that prolong the action potential duration and do cause arrhythmias (high risk). In the recent CiPA myocyte phase II validation study it was determined that compounds such as ranolazine present a problem for accurate proarrhythmia risk assessment. Ranolazine is used clinically to prolong the action potential, but is safe and does not put patients at risk for TdP. Recent studies using MEA could not properly classify ranolazine as a safe compound when using field potential duration prolongation as an endpoint. Similarly, voltage sensitive dye approaches detect ranolazine's effect to prolong APD and thus not properly classified as low risk.

Figure 18. hiPSC-CMs plated on StemBioSys' natural ECM respond to hERG block (500nM E-4031) with re-entrant (rotors) activation indicative of TdP. Each recording=10s

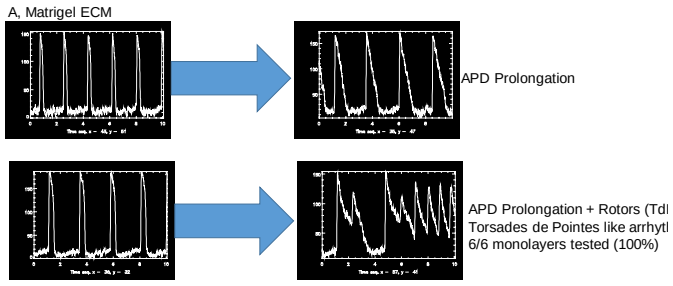
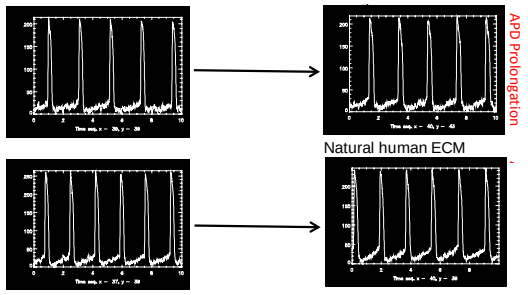


Figure 18 shows that hiPSC-CM monolayers plated on the novel natural ECM respond to 500nM E-4031 (hERG blocker) with the reproducible occurrence of rotors (TdP-like tachyarrhythmia) in 100% of monolayers tested (6/6) using fluovolt voltage sensitive dye. This is distinct from the effect observed when using matrigel ECM, where only APD prolongation was observed at the same concentration of E-4031. Next we tested the effect of ranolazine (10µM) in the same assay to determine if APD prolongation always results in TdP. Figure 18 shows that ranolazine causes APD prolongation, but not TdP type arrhythmias.

Figure 19. hiPSC-CMs plated on StemBioSys' natural ECM respond to ranolazine (10µM) with moderate APD prolongation, but not rotors (TdP). Each recording=10s



In Phase II experiments we will further validate the natural ECM in the MATURA96 platform using the full complement of CiPA validation compounds (28 compounds). We hypothesize that all high TdP risk compounds will reproducibly lead to these rotors when using natural human ECM rather than matrigel.

Phase II Approach.

Table 1. List of CiPA Validation Compounds to be used in this Phase II SBIR application

High TdP Risk	Intermediate TdP Risk	Low TdP Risk
Azimilide	Astemizole	Diltiazem
Bepridil	Chlorpromazine	Loratadine
Dofetilide	Cisapride	Metoprolol
Ibutilide	Clarithromycin	Mexiletine
Quinidine	Clozapine	Nifedipine
Vandetanib	Domperidone	Nitrendipine
Disopyramide	Droperidol	Ranolazine
D,l Sotalol	Terfenadine	Tamoxifen
	Pimozide	Verapamil
	Risperidone	
	Ondansetron	

The general approach builds upon strong preliminary data showing feasibility of the experiments and research plan. CARTOX routinely generates multi-well plates of hiPSC-CMs for proarrhythmia screening using voltage sensitive dyes. The PI has extensive experience generating hiPSC-CMs, using voltage sensitive dyes for measuring hiPSC-CM action potentials in HTS and fabricating novel cell culture platforms for cardiovascular regeneration research.[1, 2, 4, 21-23] In aim 1, the research team will fabricate and validate MATURA384 using the CiPA 28 compounds. In aim 2, the research team will develop a commercially suitable optical mapping instrument using off the shelf components and validate the system using the CiPA 28 compounds (table 1). Finally, in aim 3 CARTOX will work with StemBioSys to further validate the novel human stem cell natural ECM for use in the MATURA96 electrophysiology assay (CiPA 28 compounds).

Specific Aim 1: Miniaturize the assay to a fully validated 384-well format.

MATURA96 was validated in Phase I, and here we will extend our offerings to include 384 well plates. CARTOX has developed new methods for fabricating MATURA96 plates efficiently and will extend this

approach to also produce MATURA384. Development of a 384 well plate, fully validated using CiPA compounds, will expand the scope of CARTOX service offerings and customer base.

Experimental Design and Methods.

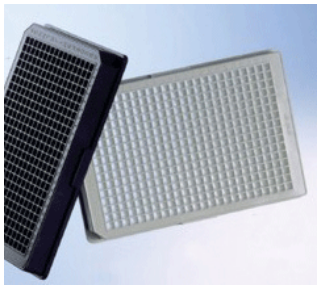


Figure 20. Bottomless 384 well plates will be developed for use in hiPSC-CM screening assays.

1.1 MATURA384 well plate fabrication. Bottomless 384 well plates (figure 19) will be obtained from VWR. Each plate will be custom fitted with PDMS sheeting to form the bottom of each well. PDMS sheeting will be obtained from Specialty Manufacturing, Inc. (Saginaw, MI) and inert silicone adhesive will be used. This sheeting has been used in the Phase I work and does not readily absorb compounds due the vulcanization process to make the PDMS inert. Each custom made plate will be sterilized using ethylene oxide sterilization. Cell viability will be quantified using live cell AnnexinV and Caspase-3/7 reagents for apoptosis detection using time lapse fluorescence microscopy (Incucyte, Essen Bioscience).

1.2 hiPSC-CM plating optimization in MATURA384. 384 well plates will be seeded with hiPSC-CMs from the same three sources that were used for MATURA96 in phase I: CDI, CLS and MCH cardiomyocytes will be plated. In each cell type, plating efficiency will be determined using the high content IncuCyte imaging instrument for live cell analysis. Initially, cell density will be the same as validated for MATURA96 ($1.56x10^5$ CMs/cm^2). This plating density corresponds to using 8,750 CMs per well of a 384 well plate. Each 384 well plate will require ~$4x10^6$ hiPSC-CMs in total. Phase contrast images will be used to monitor the extent of confluence of each well and optimal cell plating density will be determined by the monolayer confluence as described above. Additionally, dye loaded (fluo volt or calcium sensitive dye) monolayers will be used to determine the cell morphology in MATURA384 plates.

1.3 MATURA384 proarrhythmia detection validation. 384 well plates will be imaged initially using the CARTOX optical mapping platform currently in use (see figure 3 above). For each plate, 30s recordings will be made before and after application of CiPA 28 compounds. CiPA compounds are listed in table 1. Dosing of each compound will be as directed from the CiPA myocyte phase II validation study. We will determine the well to well variability as well as the plate to plate variability and ROC to assess the assay.

Timeline: This aim will be completed in the first year of the phase II funding period (0-6months). 384 well plate fabrication will begin immediately, with screening of CiPA 28 compounds being completed after 3 months using voltage sensitive dyes as well as calcium sensitive dyes. A potential caveat to consider is whether each monolayer will be of sufficient size to observe TdP activation patterns, so MATURA384 may eventually be considered as an initial front line screen for compound pro arrhythmia effects relying on APD.

Milestones for Aim 1. Significant milestones will be assessed periodically over the course of the grant period for aim 1. Significant milestones for aim 1 include: 1. Fabrication of MATURA384 well plates using PDMS sheeting and bottomless plates using biosafe adhesives. Successful sealant of plates will be determined by using alternating colored solutions (food coloring) to determine any solution mixing from well to well. We aim to generate plates with excellent seals and absolutely no solution mixing well to well. 2. Determination of the optimal hiPSC-CM plating density to generate continuous monolayers for electrophysiology testing. 3. Validation of the 384 well plates for predictive proarrhythmia screening; AUC for ROC curves screening the full CiPA 28 compounds are aimed to be ≥0.85.

Specific Aim 2: Develop an optical mapping instrument for high throughput screening electrophysiology assays using voltage and calcium sensitive dyes, with quantification of impulse conduction velocity using MATURA96 HTS. A limitation of our existing optical mapping configuration is the field of view offered by currently used lenses (Navitar, DO-2595, TV lens). Currently using this lens and set up we can only visualize a 6x6 array (36 wells) of a MATURA96 well plate at a time. Thus, mechanical translation of the plate under the CCD camera detector is required and a total of four acquisition movies are necessary per plate. In Phase II efforts we will broaden the field of view for electrophysiology data acquisition to enable recording from the entire 96 well plate at one time. This advance will simplify the experimental protocols, the data analysis and shorten even more the current experimental time of 5 minutes per plate.

A major advantage of using this VSD technology is that it is amenable to both 2D and 3D cell culture approaches. Preliminary publications have shown our ability to utilize this optical mapping approach for 2D and 3D stem cell derived cardiomyocyte experiments.[2, 23] This offers a versatility of use that will be attractive to users of the equipment. The multi parametric aspect of using fluorescent dyes is also an advantage over MEA systems which report only on the field potential.

Experimental Design and Methods.

2.1 Optical mapping instrument. We will work with Dr. Andrew Allan to build this benchtop instrument. Dr. Allan has prior industry experience in the VSD cardiotoxicity screening field working for Clyde Biosciences and Cairn Research Ltd. Thus Dr. Allan complements the PIs experience nicely and this collaboration ensures feasibility of our endeavor to build a commercially suitable cardiac hiPSC-CM optical mapping system. Figure 21 is a schematic of the system to be built. The imaging system is designed with ease of use and reproducibility in mind. A major advance over CARTOX existing system will be the ability to image an entire multi-well plate (6 well plate to 384 well plate) in one movie. Initially we will use a Scimeasure DaVinci camera 2k CMOS camera (2048x2048-114 fps; 512x512-450fps) which has specifications that are ideally suited for this aim (see attached letter of support from Scimeasure). Use of the Schnieder 60mm lens (NA=0.92) will enable visualization of an entire multi-well plate. This innovation will accelerate the rate of data acquisition significantly and also reduce potential variability due to translating plates under the camera. Another innovation is the use of a 96 well plate illuminator (470nm LEDs) for VSD illumination. In this configuration each well will be independently illuminated with its own LED to ensure even signals over the entire plate which is also predicted to reduce well to well variability. The LED plate illuminator is available from CAIRN Research Ltd, and they have evaluated our design and will be serving as collaborators on this project (see attached letter of support from Jeremy Graham). Importantly, all assay information will be logged and stored for each experiment. This data logging will be important for eventual GLP screening efforts. All components are commercially available so no major obstacles are anticipated.

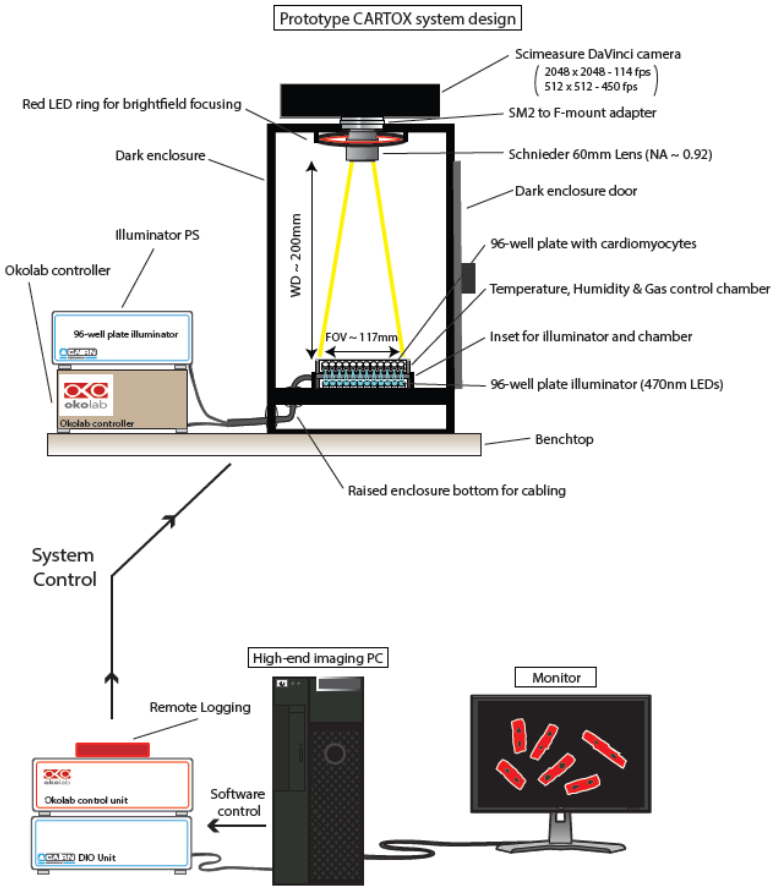


Figure 11. *Schematic of the CARTOX optical mapping system to be built and validated. All components are commercially available and will be assembled at the CARTOX laboratory.*

2.2 Instrument validation. The utility of the instrument for HTS hiPSC-CM pro arrhythmia screening will be determined using the CiPA 28 compounds in a blinded validation study. These compounds will be screened using MATURA96 with voltage and calcium transient mapping.

Timeline: This aim will be started in year 1 and initial testing/validation is expected to be complete by year 2.0.

Milestones for Aim 2. Significant milestones for aim 2 include**:** 1. Obtaining the components from each vendor. Preliminary plans have been drafted and vendors have been contacted regarding the suitability of utilization of their products for construction of the optical mapping system (see attached letters from

SciMeasure and CAIRN, Ltd.). 2. Assembly of components to form the optical mapping system and development of software for data logging and image analysis. 3. Validation of the optical mapping system using positive controls (E-4031, isoproterenol, JNJ303) and the CiPA 28 compounds. 4. Validation that the system has versatility to measure different parameters (action potentials and calcium transients).

Specific Aim 3: Optimize the use of natural, human cell generated ECM for hiPSC-CM electrophysiology screening assays. We will build upon preliminary collaborative work with StemBioSys showing that use human cell generated ECM improves the sensitivity and specificity of hiPSC-CM electrophysiology screening.

Experimental Design and Methods.

3.1 StemBioSys natural ECM coated multiwell plates. StemBioSys has started to manufacture these proprietary plates in 96 well format. This has required innovation as the manufacture requires manual handling of each plate. This process will be automated using liquid handling robots (BioTek MultiFlow FX and BioStak). To confirm manufacturability and reproducibility, 3 lots will be produced utilizing perinatal stem cells from three different donors. Composition and physical properties will be characterized using mass spectrometry and atomic force microscopy, respectively. Variability in these characteristics will be compared to StemBioSys' commercially available CELLvo™ Matrix as a benchmark. As the ultimate functional measure will be the ability of matrices to elicit desired cell-behaviors on a consistent basis, each of the 3 lots of matrix will be used to test APD prolongation and TdP arrhythmia induction following exposure to E-4031. Variability in cell response on multiple lots of matrix will be compared to variability on matrigel (current gold standard).

3.2 Validation of hiPSC-CM Monolayer Structure. The effects of natural human stem cell derived ECM on hiPSC-CM structure will be determined using microscopy approaches. First, live cell imaging will be used to monitor the transition of single hiPSC-CMs plated at high density to form mechanically and electrically connected monolayers. Second, immunofluorescent staining and confocal imaging will be done to determine sarcomere and cell-cell junctions. Sarcomere staining for α-actinin, cTnT and myosin will be done as before.[1, 2, 24] Cell-cell junction structure will be determined by immunostaining for connexin43 (Cx43), N-cadherin and plakophillin (PKP2). Antibodies for these proteins have been used extensively by the investigative team, so no major obstacles are anticipated. Confocal microscopy will be done in the UM Cardiovascular Regeneration Core Laboratory (Nikon A1R).

3.3 Validation of StemBioSys ECM for predictive hiPSC-CM proarrhythmia screening. Again the CiPA 28 compounds will be utilized for validation of natural human stem cell ECM in the MATURA96 screening assay. Validation will be done in a blinded manner with the operators blind to the identity of each compound. Compound unblinding will be done only after screening all 28 compounds, analyzing data and generating dose response curves for each compound. ROC curves will be generated as in figure 9 above and success will be measured in the AUC value. Values >0.85 are required and we hypothesize that using natural human stem cell ECM may provide values up to 0.95.

Timeline: Aim 3 will be initiated in year 1 with StemBioSys manufacturing 96 well plates for cardiotoxicity screening. Plates will be manufactured, then shipped to CARTOX for hiPSC-CM plating and electrophysiology screening. Validation using all 28 CiPA compounds as well as positive controls will be completed for the three cell types listed above and is anticipated to be completed by the end of year 2.

Milestones for Aim 3. Significant milestones for aim 3 include: 1. Automated natural ECM plate manufacture with low batch to batch variability. 2. Confirmation of hiPSC-CM complex patterning in 96 well plates. The preliminary experiments were performed using 6 well, large format plates. Miniaturization may have unexpected effects on the ECM organization. 3. Validation using CiPA 28 compounds with focus on TdP induction by intermediate and high risk compounds.