Feasibility Assessment

Ability to execute the proposed solution:

TEAM: The team assembled for this proposal have the necessary expertise and resources to successfully execute the presented solution. Dr. Pelaez has extensive experience in the construction of tissue culture bioreactors, which includes self-contained bioreactors to deliver dynamic cyclical strains (both compressive, and tensile), as well as electrical stimuli to differentiating stem cell cultures in various biomaterial scaffolds. All of these bioreactors have been designed to enhance the structural differentiation and functionality of stem cells, as is the one proposed under this solution. He and Ms. Acosta have published on the use of dynamic tensile strain bioreactors to enhance the cardiomyogenic functionality of adult neural crest-derived stem cells, as well as on the use of electrospun biomaterial scaffolds to enhance differentiation outcomes. Dr. Pelaez has further experience in stem cell biology and neurogenic differentiation from various stem cell populations. In a collaborative effort, Drs. Pelaez and Harbour have completed genomic and molecular biology characterization of over 15 primary retinoblastoma tumors collected by Dr. Harbour in his practice. This has led to the establishment of 6 primary retinoblastoma cell lines in the Harbour laboratory which are now being studied in various inter-laboratory collaborations, and which will be made available to the team as tumor positive controls when analyzing RB1 knockout iPSC cell lines in the current proposal. Dr. Harbour adds significant strength to the team with his expertise in molecular genetics, ocular oncology, and clinical background. Dr. Harbour has many years of experience in studying retinoblastoma and has published articles related to the function of the RB1 gene and protein in leading journals such as Science, Cell and JCB. As vice-chair for translational research in Bascom Palmer Eye Institute, director of the ocular oncology service, and leadership in Sylvester Comprehensive Cancer Center of the University of Miami, Dr. Harbour brings significant resources that will ensure the proposed solution receives all the support required to bring it to fruition.

MILESTONES ALREADY ACHIEVED:

- Bioreactor Chamber Prototypes: 2 prototype iterations of the bioreactor single-chambers were machined and assembled by Dr. Pelaez' laboratory from polycarbonate tubing and acrylic sheets. This was done for the team to optimize the final design, assess limitations in carrying out the working protocol proposed, and to evaluate hydrogel formulations for organoid encapsulation.
- RGC Maturation and Maintenance Media (RGCM): The media formulation proposed for inner retina maturation and maintenance has already been formulated and tested (see Appendix – manuscript in preparation). Photoreceptor (outer retina) maturation media formulations are available from literature
- Retinoblastoma Knockout (RB1KO) iPSC lines established: We have established 2 separate RB1KO iPSC cell lines in the Pelaez lab and have optimized transfection protocols for CRISPR/Cas9 gene editing in these iPS cells. RB1KO lines are already under evaluation in the retinal organoid differentiation protocols (see Appendix).
- Primary Rb Cell Lines Available: Dr. Harbour and Pelaez have 6 primary Rb cell lines available for genomic and molecular biology comparisons when positive controls are needed in the disease modeling application proposed. These primary Rb cell lines faithfully recapitulate the endogenous Rb tumors when transplanted into animal xenograft models (manuscript in preparation).

MILESTONES NEEDED AND TIMELINE:

- Final 3D Printed Build of Bioreactor Chamber Assembly: Underway. Work orders for 4 bioreactor chamber assemblies have been submitted to the University's machine shop and should be completed by the time this proposal is reviewed. Time to completion: 2 weeks (mid-August 2017)
- Oxygen Microprobe Measurements of Oxygen Gradients: Our proposed use of custom gas mixtures to establish a 2-8% Oxygen gradient across the hydrogel-organoid construct is based on our calculations of media saturation curves, and diffusion rates at the mentioned 1% (inner chamber), and 10% (outer chamber) oxygen partial pressures. We plan on using oxygen sensitive microprobes to perform actual

measurements of oxygen tensions across our bioreactor chamber interface upon completion of bioreactor assembly. Time to complete: 2 weeks (end of August 2017)

- First Retinal Organoid Production in Novel Bioreactor: We plan on using our first set of bioreactor chambers to assess retinal organoid formation and differentiation through 12 different developmental time points, including up to 120 days of differentiation when photoreceptor maturation is expected. RB1KO cells (for germline and somatic disease modeling) will be run concurrently on separate assemblies. Time to complete: 5 months (mid-January 2018)

EVALUATION CRITERIA ASSESSMENT:

Evaluation Criterion 1:

Cell Types: We propose the use of natural signaling gradients to achieve retinal phenotype differentiation. We provide segment-specific stimulation to the inner and outer retinal layers to fully develop. The use of retinoic acid and reactive oxygen scavengers for the outer retinal phenotypes, and neurotrophic stimulation for inner retinal neuron development concurrently should ensure that more adequate differentiation of all retinal neurons is accomplished.

Structure: Our proposed solution attempts to re-create how the retina naturally achieves maturation by providing retinal segment-specific stimulation to opposing sides of the developing organoids. We use bioreactor chambers to separate stimuli that are normally compartmentalized by retinal vasculature, the cavities of the eye, and the membranes lining the retina. Hopefully, this external differential stimulation, combined with the self-organizing intra-organoid modulation can achieve full retinal phenotype differentiation. Furthermore, we propose the use of hyaluronic acid hydrogels to mimic the natural environment in which the retina normally develops.

Viability: The establishment of oxygen gradients ensures that the metabolically active outer segments have the required oxygenation, while the developing inner retina is protected from neuropathic levels of oxidative stress. Similarly, the compartmentalization of unique stimuli to satisfy trophic requirements for all retinal layers (like the neurotrophic support required by RGCs to remain viable) will enhance the viability of all retinal layers through the final time point of experimentation.

Functional Characterization of Cell Types: Our bioreactor design provides the versatility to change media formulations for inner and outer retina requirements at any given time point. Our RGC maturation and maintenance media has been shown to maintain RGCs in a functional state we beyond of any reported in the literature (see Appendix).

Evaluation Criterion 2:

Robustness and Reproducibility: We have stated our willingness to make all materials used to generate this proposal available to researchers. This includes CAD drawings of our bioreactor chamber and lid assemblies for others to 3D print. We have provided a working protocol for use of the bioreactor based on our testing with bioreactor prototypes, and have provided our specific formulation of the RGC media in the Appendix. Any requests for further information will be graciously provided.

Evaluation Criterion 3:

Biology/Disease Modeling:

- What aspects of the protocol are in place to improve faithful recapitulation of biological complexity?
  - We have developed a proposal and preliminary data that support our use of RB1 knockout iPSC cell lines to reproduce the germline and somatic manifestations of retinoblastoma tumors. We will use this platform to elucidate the molecular events in initiation, RB1-dependent retinal phenotype differentiation, Rb cell of origin, malignant transformation, and progression to hypoxic adaptation of these tumors.
- How will this recapitulation be validated?
  - Our system allows us to track RB1KO cells by their expression of GFP under the native RB1 gene promoter, thus allowing us to faithfully sort these cells for downstream analysis and validation of biological complexity. Furthermore, the availability of primary human Rb tumor samples and cell

lines will allow us to comparatively assess the progression of Rb neoplasms in our retinal organoid platform to the actual pathology.

- How will viability be tested, and how is the disease state expected to affect viability?
  - RB1 inactivation by CRISPR/Cas9 is tantamount to cell line immortalization (analogous to using SV40 Large T-antigen inoculation). We have already produced these RB1KO cells which are currently undergoing the retinal organoid differentiation protocol (see Appendix). RB1 knockout produces a temporary lag in cell proliferation and a period of cellular stress during the selection process, but this phase has been overcome in our cultures.

Protection of Human Subjects:
All iPSC lines created in the Pelaez lab are generated from commercially available parent cell cultures acquired through public biorepositories and are completely de-identified. Primary Rb cell lines established in the Harbour laboratory were obtained under a University of Miami IRB approved protocol (IRB #20130636) and de-identified prior to being brought to the laboratory. There will be no recruitment of human subjects, or human subject research performed under the current proposal.

Use of Human Stem Cells:
While there is no use or establishment of embryonic stem cell lines under the current proposal, the team lead and members assure that all protocols and studies will abide by the 2009 NIH Guidelines for Human Stem Cell Research as it pertains to induced Pluripotent Stem Cells (iPSC).

Biosafety Issues:
All of the proposed studies in this solution fall under Biosafety classification level 2 (BSL2). As such, all appropriate precautions will be taken when performing the scope of work outlined. Both the Harbour and Pelaez labs have the engineering controls (biosafety cabinets, fume hoods, and area containment), personal protective equipment, standard operating procedures (SOPs), and training requisites for personnel needed to perform BSL2 work.

Intellectual Property Protection:
The presented solution includes the use of commercially available resources that may be subject to patent protection by third parties (such as the CRISPR/Cas9 reagents purchased from Origene, and the Cyto-Tune™ iPSC Reprogramming kit acquired from Thermo Fisher Scientific, among others). The solvers have no claim of intellectual property rights for these commercially available resources, and refer any matters related to their potential use for applications other than the research proposed here directly to the intellectual property owners or their licensees.