II. PROJECT NARRATIVE

Introduction: Climate change and local anthropogenic inputs often have chronic, sublethal impacts on organisms that are not detectable via conventional ecological assessments of presence and absence, or percent cover. Further, the use of ecological metrics to track impacts on organisms is reactive, not proactive. In light of the increase in chronic environmental stressors associated with climate change, essential information on organisms and ecosystem health can be ascertained via energetic measures, specifically lipids. A primary contributor to organism function is lipid content, as it is essential for all cellular membranes and macromolecules for cellular respiration. The total lipid content within marine invertebrates, such as reef-building corals, has been inversely correlated to health (Yamashiro, Oku, and Onaga 2005). In times of abundant energy production, organisms can store excess energy within their cells in the form of various types of lipid molecules (Chen et al. 2017). The lipids in turn can then be used to provide energy for times of environmental stress; for example after a marine heat wave (Grottoli, Rodrigues, and Juarez 2004), or after a fresh water driven salinity change event (Cooper, Gilmour, and Fabricius 2009). Previous studies have shown that alterations in total lipid content in corals are detectable within a few weeks following environmental perturbations. However, detailed analyses of real-time lipid depletion and the associated biological effects have not been reported for live corals. Current protocols for measuring lipid content include lengthy specimen preparation with toxic reagents and cost-prohibitive instrumental analysis in the laboratory (Saunders et al. 2005). *Here, we propose the implementation of a rapid, non-invasive lipid biosensor that could be used in both proactive and long-term monitoring applications related to coral health.*

Significance and Innovation: We have developed a near-infrared (NIR) fluorescence nanosensor, based on the intrinsic optical properties of single-walled carbon nanotubes, that has been shown to enter individual eukaryotic cells and report the total lipid content of the cultured cells and live animals (Jena et al. 2017). Our carbon nanotubes optical reporter (CNOR) lipid biosensor (1nm diameter x 100nm in length), is easily taken up through active endocytic processes (Bhattacharya et al. 2012). Once internalized in mammalian cells, the nanotubes remain within the endolysosomal vesicles (e.g. endosomes and lysosomes), pose no detectable harm, and can ultimately be cleared from the cells (Gravely, Safaee, and Roxbury 2019). The fluorescence emission of nanotubes is indefinitely photostable and reversibly responds to enable the real-time and dynamic assessment of lipid content. Additionally, the NIR fluorescence signals from CNOR can be obtained through up to 1 inch of biological tissue. Collectively, these properties of our CNOR lipid biosensor enable long-term, live, *in situ* measurements of intracellular lipids. To date, we have deployed this technology for the detection of various lipid-related human diseases in live cells and animals, including Non-Alcoholic Steato-Hepatitis (NASH) and Niemann Pick Type C (NPC) (Galassi et al. 2018).

Objectives: The objectives of this proposed project are to lay the foundation for quantitative applications of carbon nanotube optical reporter (CNOR) lipid biosensors to address critical questions in coral biology. We specifically propose to:

Objective 1. Determine the optimal exposure time, concentration, and potential toxicities of the CNOR lipid biosensors within various coral species.

Objective 2. Identify the mechanism of uptake into the coral cells and precise subcellular localization of the CNOR lipid biosensors.

Objective 3. Determine the fate of the CNOR lipid biosensors after cellular internalization.

Preliminary Data: Through initial highly collaborative experiments between Putnam (CELS, coral biologist) and Roxbury (Engineering, chemical engineer), the uptake of the CNOR into the corals (Fig. 1A) was quantified non-invasively and non-destructively with a NIR probe spectrometer (Fig. 1B). The spectra indicate that the CNOR fluorescence resides in a distinct spectral region (1050 - 1350nm) and can be deconvoluted from algal autofluorescence (Fig. 1C).

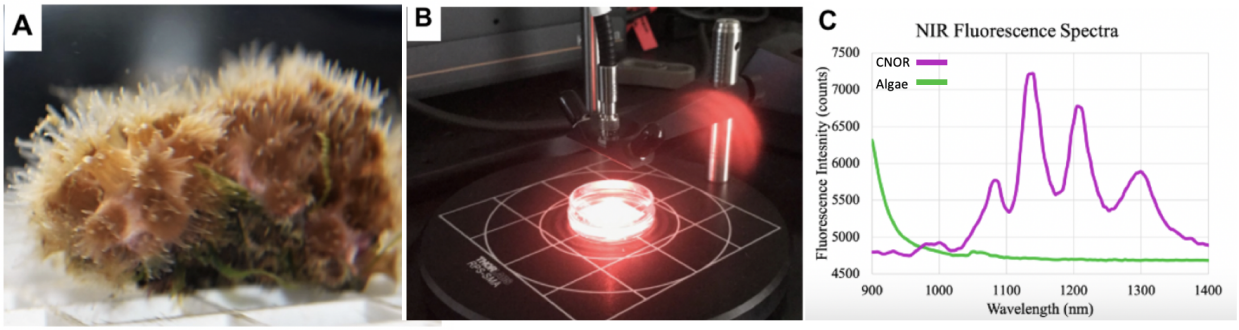

Figure 1. CNOR uptake in A) the northern star coral, *Astrangia poculata*, can be quantified B) live via a NIR probe, and C) separated from algal autofluorescence in the NIR.

Additionally, NIR fluorescence images were obtained from coral tissues after a standard histology preparation (Fig. 2). The images suggest that the CNOR (**red**) are internalized by the gastrodermal cells of the corals containing autofluorescent symbionts (**green**) and again can be spectrally deconvoluted from one another.

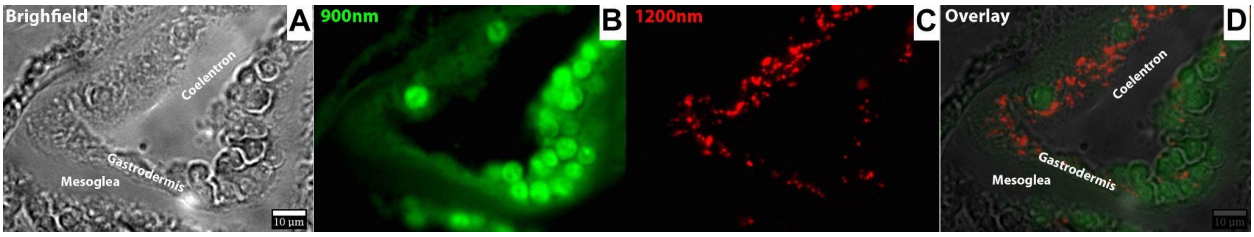

Figure 2. A) Brightfield microscopy of histological sections of the coral *Pocillopora acuta* scanned with a NIR microscope reveal the presence of B) coral autofluorescent symbionts (**green**), surrounded by C) CNOR (**red**) lipid biosensor signal in the areas of previously documented lipid body accumulation (Chen et al. 2021) in the D) gastrodermal tissue.

Importantly, our newly-developed method of assaying lipid content is inherently non-destructive and enables the real-time, *in situ* monitoring of lipid content, which we know is critical to coral survival. *Our CNOR will give environmental scientists unprecedented temporal resolution to detect sublethal stress in live corals and thus provide proactive, scientifically-informed management that can be extended to other marine invertebrate indicator species.*

Work Plan: Objective 1. Corals (*Pocillopora spp*, *Acropora spp*, and *Astrangia spp*) are currently maintained in the PI's laboratory under standard growth conditions. Small coral fragments will be added to a 6-well plate and subjected to varying solutions of CNOR. The time of exposure and concentration of CNOR in the incubated solution will be varied by orders of magnitude (e.g. 1 minute → 24 hours and 0.1 → 10 mg/L, respectively) in order to determine the parameters that gives rise to the optimal rate and cumulative mass of uptake into the corals, as determined by NIR fluorescence intensity, while limiting any potential toxic effects. Toxicity will be determined by Trypan blue staining of dead cells (Roger et al. 2021), and quantification of polyp expansion and responsiveness (Burmester et al. 2018). These assays are common in Putnam's lab.

Objective 2. A series of assays will be employed in order to determine the mechanism of uptake, subcellular localization, and long-term fate of the nanotubes. First, in accordance with previous studies on mammalian cells (Jena et al. 2017), a visible fluorescence co-localization assay will be performed with Lysotracker Red (Invitrogen) and FITC-tagged nanotubes to confirm uptake through the endosomal pathway of the coral cells. Upon internalization of the nanotubes into the intact corals, individual polyps will be treated with the lysosomal dye and confocal fluorescence images will be acquired. The degree of co-localization between Lysotracker and nanotubes will be quantified using Pearson correlation coefficients. Next, the spatial distribution of nanotube mass throughout the coral cells will be quantified with the use of confocal Raman microscopy. Here, the "G-band" intensity of the localized nanotubes can be converted into a weight, resulting in the construction of a spatial map of nanotube mass within the coral cells (Gravely, Safaee, and Roxbury 2019). These assays are common in Roxbury's lab.

Objective 3. Finally, the long-term fate of the internalized nanotubes will be examined through extended NIR fluorescence assays using the probe spectrometer. The corals will be incubated in nanotube-containing seawater for one hour followed by a thorough washing and placement back in fresh seawater. The corals will be placed under a probe spectrometer, and the NIR fluorescence will be examined daily for a duration of at least one week. If the nanotube signal remains after one week, the study will be extended.

Short- and long-term benefits to the faculty member's research program: Both Putnam and Roxbury began at URI in 2017 and have established and continuously growing programs. We are working on a peer-reviewed perspective outlining the enhancement of coral health via nanotechnology and bioengineering. This proposed work represents a new interdisciplinary research direction for both PIs within a field with ample opportunities for growth.

Plan for Submission of Extramural Proposals: We previously proposed to use the CNOR as an environmental monitoring tool for a RI STAC call, but the reviewers recommended more preliminary data was needed to support its field application capacity. Therefore we have used our startup resources and personal time to generate our preliminary histological evidence of CNOR uptake and stability. Our current data and resources are however insufficient to move this innovative project forward. Funding from the URI Proposal Development Grants Program would provide the rigorous preliminary data to support full proposals to national funding agencies such as NSF and NIH. Our first two planned submissions of full proposals will be to 1) NSF Biosensing and 2) NSF Integrative and Organismal Systems (IOS). Both of these programs accept proposals throughout the year, providing us the chance to build clear and strong preliminary data to enhance our chances of a successful NSF award.

Timeline and Milestones

	2022						**2023**					
	7	**8**	**9**	**10**	**11**	**12**	**1**	**2**	**3**	**4**	**5**	**6**
Objective 1												
Objective 2												
Objective 3												
Proposal Submission												