Deciphering Microbial Structures *in situ* Using Subcellular RNA Sequencing.

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Project Goals:

This study aims to develop methods for characterizing microbial communities with single-cell and spatial resolution. We are employing fluorescent *in situ* sequencing (FISSEQ) technology for acquiring genomic and transcriptomic information to better understand the spatial arrangement of microbes and their gene expression in natural and synthetic microbial communities.

Most investigations reduce microbial physiology to monoculture conditions, which does not consider their abundant interactions in natural environments. Indeed, much of our understanding of microbes stems from gene deletions, heterologous expression, and *in vitro* enzyme characterization. Microbes need to be studied *in situ* where their spatial organization holds biological importance for responding to the environment (*e.g.*, polymicrobial metabolism, biofilms, horizontal gene transfer). However, *in situ* characterizations of these complex phenotypes in natural environments are generally not feasible and remain largely unknown.

Microbial cell walls and membranes are notorious for their low permeability to all molecules, such as oligonucleotides and enzymes needed for subcellular sequencing. We envision that methods for removing the cell wall and cell membrane will enable spatial characterization of a wide-range of microbes that are normally recalcitrant to single-cell techniques (e.g., fluorescent *in situ* hybridization (FISH), subcellular RNA sequencing). The Church Lab in collaboration with Ed Boyden's lab at MIT have developed Expansion FISH and subcellular Expansion Sequencing (ExSeq), based on the Expansion Microscopy (ExM) technique. ExM is a super-resolution imaging technique whereby biological samples are embedded in an acrylamide/acrylate hydrogel and physically expanded (in ExSeq; ~3.5x linear expansion, ~40x volumetric expansion).

Here, we describe our progress towards subcellular sequencing of microbes using FISSEQ paired with ExM. We have found conditions for performing ExM on *E. coli* and *Saccharomyces cerevisiae* and find that cell wall degradation is required for the successful expansion of cellular components. Further, we have developed a series of chemical treatments that covalently attach total cellular nucleic acids to the ExM hydrogel, which is a pre-requisite for generating sequencing libraries while maintaining their original spatial location. Notably, we establish protocols for generating *in situ* sequencing libraries that enable multiplexed assays for identifying microbial taxonomy and gene expression, and demonstrate preliminary results for whole-transcriptome sequencing.

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