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PNNL-32901

Multiplexed Probing for Functional Capacity Measurements in Complex and Limited-Size Samples

June 2022

Aaron T. Wright
Susan Ramos-Hunter
Natalie C. Sadler

U.S. DEPARTMENT OF ENERGY

Prepared for the U.S. Department of Energy
under Contract DE-AC05-76RL01830

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Pacific Northwest National Laboratory
Richland, Washington 99354

Abstract

A new strategy using glass as a solid support for functionalization with chemical probes has been recently developed with successful results. The next step in glass functionalization is to pair chemical probes with fluorescent glass microspheres. This approach gives us a way to directly quantify probe-bound protein using Fluorescence-Activated Cell Sorting (FACS). FACS has already shown to be amendable to glass microspheres, demonstrating changes in probe-bound protein concentration. Suitable probing conditions for gram-positive and negative microbes, complex microbial communities from myriad ecosystems, and eukaryotic cells/tissues have traditionally suffered from set-backs, such as limited protein per sample and conditions atypical for probing. To remedy this, we propose to:

(1) Demonstrate probe functionalization specific to glass microspheres by paired fluorophore and use those activity probes successfully with proteomics. Probes that are currently available (CYP5, GSH/GST, Glycoside Hydrolase) can be easily prepared onto glass surfaces. Probe-bound microspheres can be tested in ratios, first with known amounts of purified protein and then complex microbiome lysates.

(2) Our validated microspheres are then used to determine quantitative amounts of protein through FACS sorting. Complimenting these results by proteomics, prepared microspheres are then optimized and condensed into a user-friendly kit.

Once successfully commercialized, this multiplexed assay can be used with little to no technical expertise, making this available to various industries users.

Summary

The goal of this proposal is to develop a proof-of-concept technology that is commercially deployable, can be applied to clinical, pharmaceutical, or agricultural-based proteomic analysis in a user-friendly assay format. Currently existing activity-based probes are leveraged by functionalizing fluorescent microspheres' surfaces. Multiplexing the probe-fluorophore microspheres in gut or soil microbiome lysate decreases the need for large sample amounts and labor intensive, traditional proteomics. The protein-probe-fluorescent microspheres are sorted and quantified by using FACS. This method enables us to make initial quantification measurements of complex microbiomes and out of that milieu, direct probe-sorted proteomics analysis. Proteomics benefits from the development of the microsphere fluorescence-driven sorting, as protein-probe-microsphere groups can be isolated and analyzed discretely using mass spectrometry.

Acknowledgments

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Acronyms and Abbreviations

FACS – Fluorescence Activated Cell Sorting

PEG – Polyethylene glycol

MAS – Magic-angle spinning

NMR – Nuclear magnetic resonance

GFP – Green fluorescent protein

TGA – thermal gravimetric analysis

FISH – fluorescence in situ hybridization

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1.0 Introduction

There is a growing research interest in avoiding characterizing microbial communities by genomes or transcriptomes alone. However, current techniques using fluorescence in situ hybridization (FISH) for sorting microbes from microbiomes based upon gene content still almost universally fail to provide a sorting mechanism based solely upon function. This technique, and many others, are based upon labeling of genes or amino acids; however, the presence of a gene or an amino acid does not necessarily equal function. New techniques are needed that identify, separate, and quantify analyte species (for example, microbes, enzymes, toxins, and the like) present in biological environments (for example, soil, water, air, cells, and the like) so that such species and their functions can be determined.

2.0 Results

The function-based probes disclosed herein can be deployed directly in living microbial populations within their native habitat. The probes are capable of binding to analytes of interest and even can be taken up by cells. The probes are designed with specific structural motifs that facilitate interactions with a particular analyte such that the particular analyte will bind the probe while other analytes will not. The probes further comprise structural components that facilitate binding to the analyte and detecting the probe-analyte conjugate. The probes thus can be used to detect the presence of particular analytes and further can be used to enrich these analytes for downstream analysis (for example, flow cytometry or other detection methods, sequencing, proteomics, and combinations thereof). A schematic illustration of methods that use certain probe embodiments described herein is provided in FIG. 1. In particular embodiments, the probes are used to functionally annotate and isolate uncultivated microbes present, for example, in cellular environments, soil environments, aquatic environments, and combinations thereof.

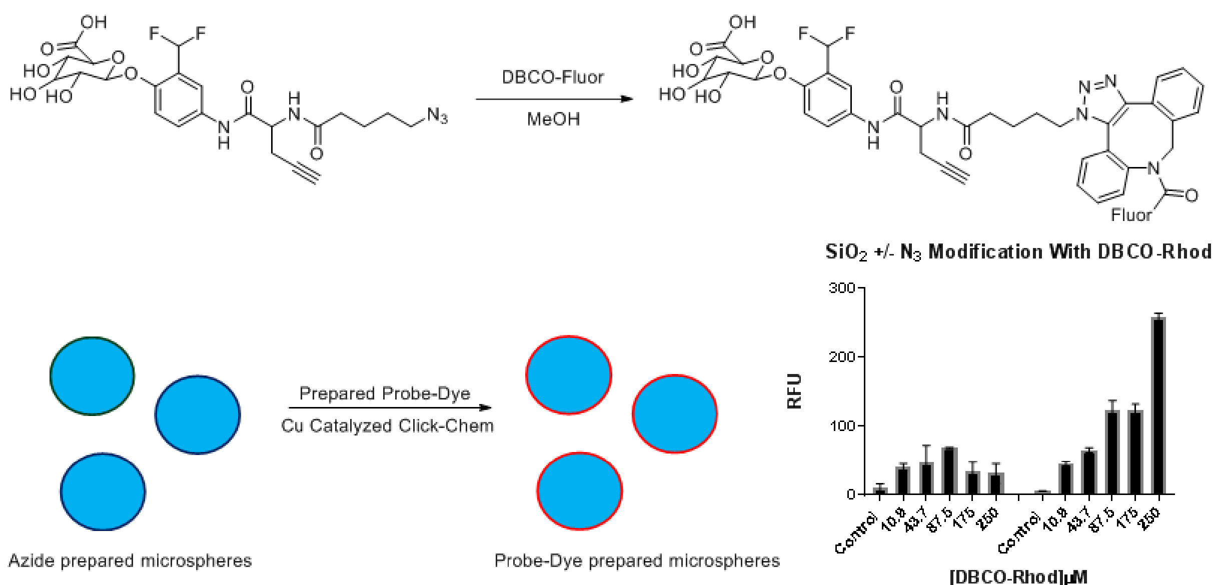


Fig.1. Workflow and analysis for microsphere modification SiO2 microspheres were purchased and treated with concentrated acid (80oC) to remove organic impurities and prepare for silane functionalization. Microspheres will be fluorophore linked and coupled to a functional group linker (PEG or aliphatic chain). Linker prepared surfaces will then be characterized and quantified by Solid-State Magic Angle Spinning (MAS) NMR and Thermogravimetric analysis (TGA) respectively.

Probe embodiments described herein comprise moieties that facilitate their use in function-based sorting and identification of analytes present in environmental microbiomes. In particular embodiments, the probes comprise a reactive group capable of forming an irreversible chemical bond with a target analyte. The reactive group forms a stable covalent bond with analyte species in complex proteomes upon exposure to a reactive region of the analyte (for example,

protein residues) and/or by activation of the reactive group to form a reactive intermediate that binds to the analyte (for example, photoreactive crosslinking with a protein). In some embodiments, the probes further comprise a precursor moiety ("pTag") that can be converted to a tag moiety. In other embodiments, the probes can comprise a pre-installed tag moiety. The tag moiety (whether pre-installed or added subsequent to exposing the probe to an analyte) provides the ability to rapidly and sensitively detect and measure labeled analytes. In some embodiments, the probe can further comprise a binding group, which can be a functional group or a molecule that is attached, either directly or indirectly, to the reactive group of the probe. The binding group is used to draw the analyte to the probe. In some embodiments, the binding group can be cleaved or displaced from the probe by the analyte. Thus, the binding group can facilitate binding the probe and the analyte together via the reactive group of the probe. In yet additional embodiments, the probe can comprise an anchor group that facilitates immobilization of the probe on a substrate for analysis.

Methods of using the probe embodiments described herein also are disclosed. In particular disclosed method embodiments, the probe can be adhered to a support or simply combined with a sample. The probe is allowed to interact with any suitable enzymes present in the sample and/or can be affirmatively activated to facilitate binding the probe to the species of interest. The species that becomes labeled with the probe can be enriched and measured by proteomics, and/or can undergo further analysis (for example, imaging, SDS-PAGE, or fluorescence-activated cell sorting (or "FACS"), mass spectrometric analysis, proteomics, or combinations thereof). Methods of use are described in more detail herein. Classes of probe embodiments of the present disclosure are seen in Figure 2. Fluorescent profiling of concentration dependence is provided in Figure 3.

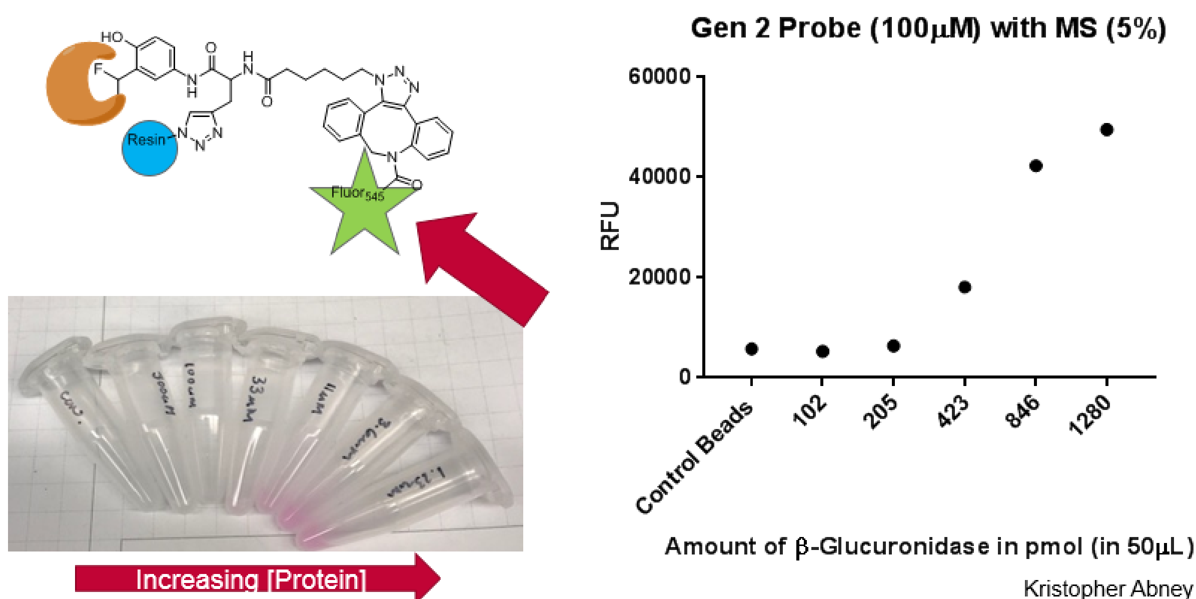
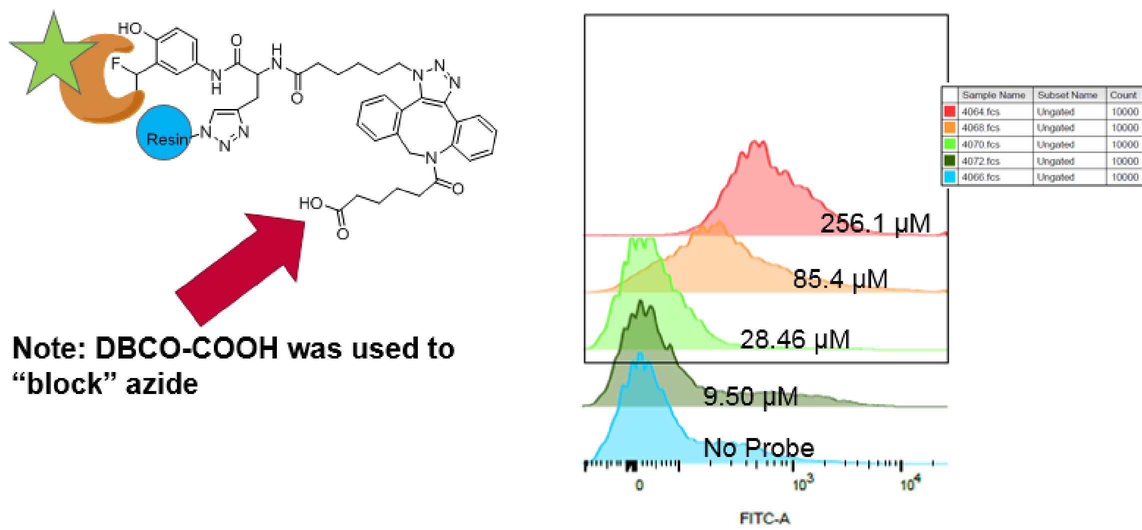


Fig. 2. Microspheres are Fluorescent after Clicking with Fluorophore-Conjugated Purified β-Glucuronidase.



Chris Whidbey

Fig.3. FACS detection of GFP-tagged β-glucuronidase clicked to microspheres increases with protein concentration

3.0 Conclusion

A new strategy using glass as a solid support for functionalization with chemical probes has been recently developed with successful results. The next step in glass functionalization is to pair chemical probes with fluorescent glass microspheres. This approach gives us a way to directly quantify probe-bound protein using Fluorescence-Activated Cell Sorting (FACS). FACS has already shown to be amendable to glass microspheres, demonstrating changes in probe-bound protein concentration. Suitable probing conditions for gram-positive and negative microbes, complex microbial communities from myriad ecosystems, and eukaryotic cells/tissues have traditionally suffered from set-backs, such as limited protein per sample and conditions atypical for probing. To remedy this, we propose to:

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Pacific Northwest
National Laboratory

902 Battelle Boulevard
P.O. Box 999
Richland, WA 99354

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