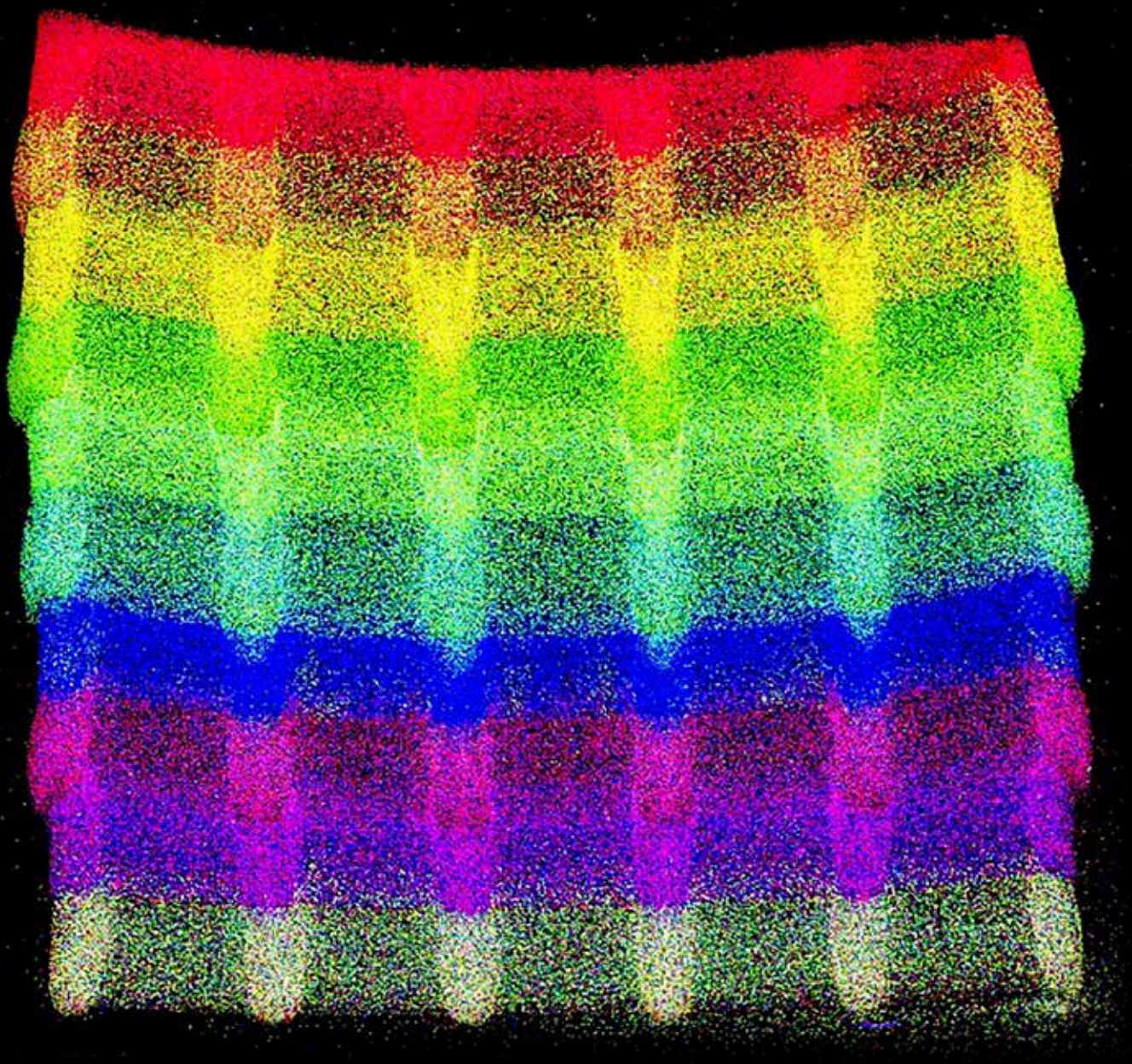
3D-Printed Microbes **ENHANCE BIOMATERIALS**



500 micrometers

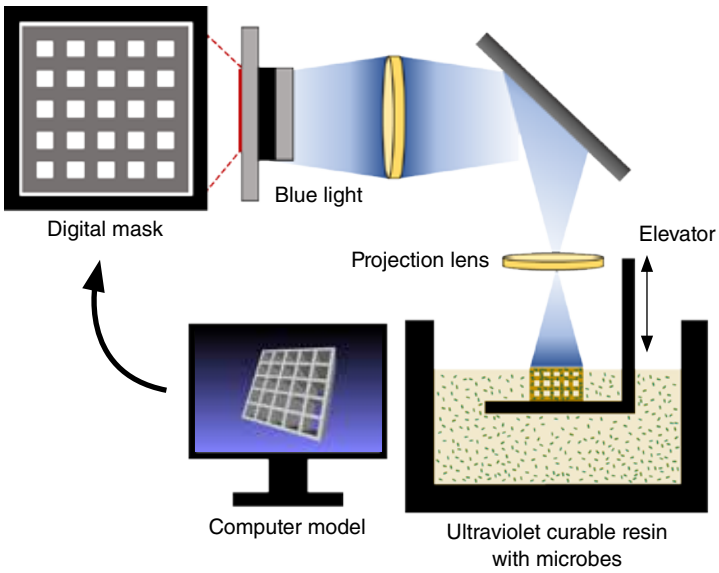
DESPITE their size, microorganisms represent a majority of biomass on Earth. Most microbes are contained in biofilms—thin accumulations of opportunistic microorganisms that form on virtually any surface that harbors basic ingredients for survival, whether the hull of a ship or the hard-to-reach spaces between one's teeth. Far from motley clumps of microbes, these microcities boast elaborate, interconnected ecosystems whose collective behavior drives fundamental biogeochemical reactions.

From a human standpoint, however, biofilms are often intrusive and pathogenic. Persistent accretions of microbes rapidly colonize medical devices, developing antibiotic resistance along the way and jeopardizing patient health. They also coat fresh produce, create food waste, and glaze surfaces of industrial machinery and transport vessels, adding billions of dollars to shipping costs. A multidisciplinary effort at Lawrence Livermore is underway to better understand the dynamics of microbiome differentiation and how certain biological processes might be harnessed through additive manufacturing (AM).

Staff engineer William "Rick" Hynes heads an exploratory research project investigating how microbial growth is tied to host-surface properties. "In the wild," explains Hynes, "microbial colonies develop their own structures through a mix of competition and symbiosis, so species partitioning and geometry come naturally; behavior and growth are a direct result of their orientation in space." Understanding the growth pattern of a lone species in a petri dish may not be too challenging, but biofilms that harbor tens or hundreds of species in unique arrangements exhibit complex behaviors which are difficult to parse, much less predict.

To understand how biological relationships within biofilms (and other microbiomes) are affected by spatial geometry, Hynes's team is utilizing the 3D printing process of biological projection microstereolithography (BioPμSL) to build intricate, living structures for studying and directing microbial growth. "Typical 3D-printing processes require melting a thermoplastic polymer and tracing out the desired shape layer-by-layer as it cools," explains Jesse Ahlquist, staff engineer and co-technical lead. "That method does not work when live cells are embedded in the material because exposure to high temperatures would kill them." As part of Livermore's Laboratory Directed Research and Development Program, Hynes's team has developed a customized stereolithographic apparatus for microbial (SLAM) bioprinting that produces complex structures at higher resolution than other microscale methods while sustaining living organisms.

This confocal image of 3D-printed grid structure (left), viewed from the side, consists of two different, alternating molecular weight bioresins. Each layer contains differing ratios of microbe-sized, polymeric-fluorescent beads to achieve 10 different colored sections, each 500 micrometers tall, in a single build.



Overview of the biological projection micro stereolithography (BioPμSL) system used to produce printed structures with embedded microorganisms.

Reciprocally Enabling Bacteria

Developed at Livermore's state-of-the-art Micro Nano Bioengineering Laboratory (MNBL), the group has already used SLAM bioprinting to generate complex lattices and whorls that can accommodate over a dozen bacterial species. With these sophisticated structures, researchers will be able to conduct controlled experiments to extend basic scientific understanding of microbial interaction from the flat, unnatural setting of Petri dishes to dynamic, 3D environments that reflect natural patterns.

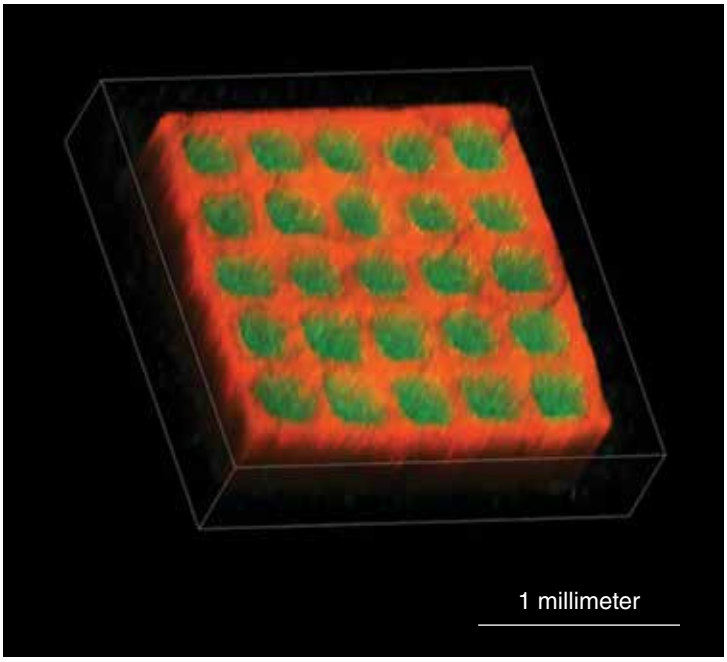
Artificial biofilms are not new, but until now, efforts to characterize the biodiversity and community stability of micro-environments have been constrained by lack of control over growth geometry. Microbes systematically grown on the same agar resort to interspecies competition over nutrients rather than expand in new directions to exhibit colony spacing and nuanced biological relationships. Exerting precise control over microbial cultures in increasingly complex settings will reveal insights into behaviors that have so far eluded researchers. "We're developing the tech that synthetic- and microbiologists don't even know they need yet," says Hynes. "We're not just trying to mimic the geometry and growth found in the wild, but as we come to better understand natural microbial behavior, we can begin thinking about functionalization, which can often require division of labor within cellular communities and can hinge on their structure."

"This is where things get really interesting," says Ahlquist. "We can use the waste products from one species to power the

processes of another. By linking different layers and different species, we achieve a consortium of reciprocally enabling bacteria." Microbes make ideal components in emerging technologies because they function in ways manufactured products cannot.

Harnessing microbes' natural metabolic processes will hasten the development of new technologies across diverse fields. By exploiting special metabolic processes—oftentimes via fermentation—bioplastics and energy-rich biofuels such as ethanol and biodiesel could be more efficiently derived from biomass, relieving dependence on finite petroleum resources. Encasing cells in printed bioresins could effectively capture and neutralize pollutants as part of bioremediation efforts in contaminated areas. Similarly, printed microbial structures could be used to absorb and purify rare-earth elements during mining operations, a capability that promotes resource independence for substances critical to U.S. defense technologies. Microorganisms even show promise as remote chemical biosensors by detecting carefully monitored substances like uranium. Any of these nascent applications' success, however, requires deeper understanding of microbial biology and behavior.

While Hynes aims to equip scientists with tools to probe these dynamics, staff engineer and co-technical lead Javier Alvarado is working on other applications. Alvarado, who studied neuroscience and tissue engineering, sees great potential for Livermore's bioprinting research in medical innovation. "One of the leading causes of infection is invasive cells' ability to form biofilms and develop antibiotic resistance," he explains. Once pathogens infect an individual, their quorum-sensing ability allows them to lurk within biofilms, evading immune detection until their numbers are sufficient to mount a full-scale attack. "Putting an artificial construct into a body, for example, is risky because there's uncertainty about how it will hold up in a new environment. Having precise control over cell growth parameters reveals how biofilms behave in different settings and how to disrupt their formation."



Oblique view of a printed grid structure containing *Escherichia coli*, which express either green fluorescent protein or mCherry in red.

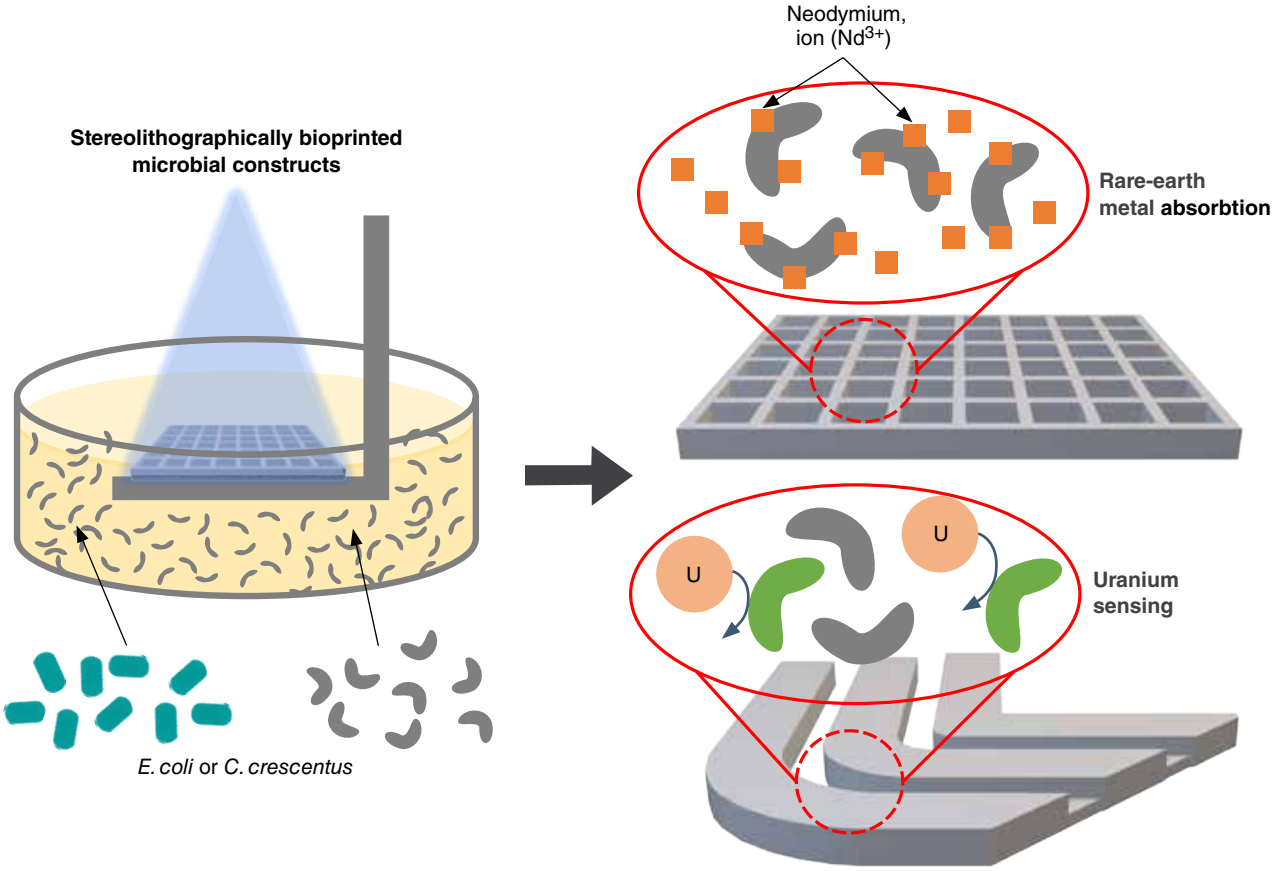
A printed version of the Laboratory's logo harbors *C. crescentus* within the material (left). The suspended bacteria fluoresce upon exposure to 10 micromolar uranium, brightening the construction (right).

An Ideal Molecular Structure

Ordinary stereolithography functions by selectively exposing layers of photosensitive liquid resin to ultraviolet light, which cures it into the desired shape. Slices of a 3D image are serially projected onto the resin, and as the top layer solidifies to form a translucent polymer hydrogel, a mechanical stage lowers the hardened layer to just below the liquid surface. Subsequent projections cure layer after layer of resin until the entire object is produced. In the case of SLAM bioprinting, the resin begins saturated with microbes, then a 405-nanometer wavelength light hardens the bioresin into hydrogel, and the organisms become living parts of the structure.

Hynes explains that SLAM Bioprinting itself is not a large-scale manufacturing technique. "It serves as a rapid prototyping method to test cells under different conditions," he clarifies. The technique will enable researchers to realize more efficient, economical, and environmentally friendly processes once scaled.

Refining large-scale production methods demands technical sophistication at the research level. For instance, wieldy



The BioPμSL printing process can encapsulate multiple bacterial species and lends itself to numerous functionalities.

microbes favored by bioengineers, like *Rhodopseudomonis palustris*, which Alvarado dubs "the Swiss Army knife of bacteria," require an electron source to synthesize bioplastics. Providing electricity to bioprinted architectures necessitated embedding carbon nanotubes inside the printed structures, a challenging task given the sensitive manufacturing method.

Ahlquist utilized MNBL's capabilities to help develop an ideal microbe-bearing resin for bioprinting experiments, ensuring compatibility between the cells and the resin's conductivity. "The process is essentially a funnel," he explains. "We look at all the possible materials we could use, and then narrow it down to one that keeps microbes healthy and performs well during the printing process." Bioresins come in a spectrum of formulations, each providing a unique combination of porosity, stiffness, molecular weight, and other properties that must harmonize for cells to thrive. The team identified a blend of polyethylene glycol diacrylate with an ideal molecular structure for cells to take root, absorb vital nutrients, and dispel metabolic waste products through passive gradient diffusion.

Ahlquist and Hynes credit Lawrence Livermore's new additive manufacturing (AM) facilities with making their work possible. "MNBL was finished just as I started at Livermore, giving us access to a breadth of AM capabilities and expertise," recalls Hynes. "This would have been incredibly difficult to pull off in academia, but at a national laboratory so many tools are readily available." For Alvarado, the Laboratory's exceptional capabilities go beyond instrumentation. "It's the people. Having mentors and mentees with diverse backgrounds makes it possible to investigate these emerging fields. Nature is the best engineer, and we're still figuring out all of its tricks."

—Elliot Jaffe

Key Words: Additive manufacturing (AM), bacteria, biofilm, biological projection microstereolithography (BioPμSL), bioplastics, bioprinting, Micro Nano Bioengineering Laboratory (MNBL), *Rhodopseudomonis palustris,* stereolithographic apparatus for microbial bioprinting (SLAM).

For further information contact Rick Hynes (925) 423-5465 (hynes2@llnl.gov).