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Title: Metaoptics Enabled Multifunctional Imaging
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Abstract: Despite the fact that most microorganisms occupy two- and three-dimensional space in heterogeneous arrangements and in proximity to other microorganisms of different species, much of our current knowledge about their metabolic processes is derived from homogenizing, then analyzing, the chemical content of cultures containing a single microbial species. This project addressed this fundamental dichotomy by developing a set of optical imaging principles combining multiple new technologies. The imaging strategies developed in this project combine metaoptics structures with active *in situ* nanoscale control of the chemical environment and applied them to a microbial system, *Myxococcus xanthus*, with particular relevance to the DoE mission. The combination of metaoptical architectures and nanoscale control over the molecular environment enables: (a) precise control over the electromagnetic (EM) field at length scales smaller than the wavelength of light; (b) control of the interaction of the EM field with critical molecular systems in DOE relevant microbes; (c) control over the chemical environment – especially the presence and quantity of reactive oxygen species (ROS) that can affect redox homeostasis; and (d) the ability to ask new kinds of questions not accessible to ‘omics’ approaches or standard methods of biological imaging. These capabilities are applicable to detailed studies of metabolic pathways in microbes and to lignocellulosic biomass deconstruction. To accomplish these objectives, we pursued two over-arching technical goals: (1) the development of new metaoptics-enabled approaches to imaging and spectroscopic characterization; and (2) the development of tools to control the chemical environment of a microbial sample with nanometer-scale precision. Goal 1 was addressed through the design, fabrication, and characterization of new metasurfaces capable of super-resolution imaging through extreme confinement of the optical field. Goal 2 was addressed by controlling the redox potential on the nanoscale in microbial communities and characterizing their effect on intrinsic bacterial fluorophores which act as molecular sentinels and through characterization of soluble factors secreted by *Myxococcus xanthus* by confocal Raman imaging. The optical imaging/sensing approaches developed here make it possible to use these powerful new imaging and sensing modalities in metabolic studies by making it possible to visualize and track the spatial and temporal expression patterns of natural or engineered pathways in microorganisms.

Research Objectives: Development of enhanced imaging tools pursued in two technical goals: (1) the development of new metaoptics-enabled approaches to imaging and spectroscopy; and (2) the development of tools to control the chemical environment of a microbial sample with nanometer-scale precision.

Accomplishments Summary: Access to publicly available scientific and technical information (STI) is through the scientific literature and is given with the summaries below. References are made to the Products list at the conclusion of this section. All references include digital object identifier (DOI) numbers.

(1) *Metaoptics.* Work in this area began by pursuing two main themes: (a) confining the plasmonic modes which exhibit hyperbolic dispersion on finite silver antenna nanostructures for use in localized plasmonic structured illumination microscopy (LPSIM) in order to characterize the resolution enhancement achievable through LPSIM with hyperbolic metasurfaces; and (b) the fabrication of metasurfaces made of 1-D gratings on thick silver films and the characterization of these computationally and experimentally (ellipsometry). Computations showed that adjusting the height, width, and periodicity of metasurface ridges results in metasurfaces that display hyperbolic dispersion in water. Next, we studied the conditions needed to confine this hyperbolic mode onto finite nanoantenna structures for use in LPSIM. We used Fourier analysis to study resolution enhancement compared to plasmonic structured illumination microscopy (PSIM). By adjusting the length of the antennas we showed that fields could be produced exhibiting an integer number of anti-nodes along the top of the nanoantennas, with peak-to-peak spacing less than that of surface plasmon polaritons (SPPs).²

Metasurfaces composed of 1-D Ag gratings on Ag films were fabricated on Si by e-beam lithography and liftoff. Metasurfaces were optically characterized by variable angle spectroscopic ellipsometry and were compared to Finite Difference Time Domain (FDTD) calculations at 45° incidence. Positions of three fundamental modes at 400 nm, 650 nm and 1020 nm and were corroborated by FDTD and were attributed to edge plasmon, localized surface plasmon (LSP) and surface plasmon lattice resonance (SPLR), respectively. The edge plasmon mode at high energy is particularly interesting for imaging, because it displays hyperbolic dispersion. In addition, two low energy mode at 650 nm and 1020 nm were marked for further study due to their resonant behavior.

In order to confine hyperbolic modes spatially, we fabricated finite length nanoridge arrays with subwavelength spacing in both axes. The large array efficiently couples free space light to multi-plasmon modes of the nanoridge.³⁻⁵ These multi-plasmon modes are engineered to exhibit a large effective modal index at 458 nm. Angle- and polarization-dependent spectroscopic ellipsometry were employed to show the measured transverse magnetic reflectance with the incident wavevector along the long axis of the ridge. For an incident angle around 30°, two higher order modes hybridize, giving rise to a coupled mode, which exhibits hyperbolic dispersion. The confined nature of the hyperbolic mode enables super-resolution imaging, as we showed numerically for a random distribution of quantum dots ($d = 9$ nm) with deep subwavelength spacing. Structured illumination microscopy (SIM) reconstruction using these metasurfaces produced images with 3.44x resolution enhancement compared to the diffraction limit, giving a resolution of 52 nm at $\lambda = 458$ nm; a result superior to other state-of-the-art approaches in SIM.

The work just described demonstrates that plasmonic hyperbolic metasurfaces are an effective platform for manipulating the propagation of light. We developed confined modes on arrays of silver nanoridges that exhibit hyperbolic dispersion and used these to demonstrate and model a super-resolution imaging technique based on structured illumination microscopy (SIM). A spatial resolution of ~ 75 nm at 458 nm was obtained, which is 3.1 times better than an equivalent diffraction limited image. This work emphasizes the ability to engineer the properties of confined optical modes and to leverage those characteristics for applications in imaging. We expect this work to lead to improved approaches for super-resolution imaging using designed sub-wavelength structures that are applicable to many problems of interest to DOE.

(2) *Myxococcus xanthus*. Potential-dependent dynamics of the bacterium *M. xanthus* were studied using combined surface-enhanced Raman microspectroscopy and electrochemistry.^{1,6} *M. xanthus* exhibit a well-characterized behavior under constrained nutritional conditions (low metabolic state), in which individual cells coalesce into larger super structures termed fruiting bodies. Remarkably, before our work there was little physicochemical or spectroscopic characterization of these structures. We began by characterizing the difference between individual cells and fruiting bodies by Raman on microspectroscopy. While potential did not affect the formation of dormant fruiting body spores, the Raman bands from 1500 to 1600 cm^{-1} showed clear potential-dependence, reflecting redox activity of flavoproteins in the outer membrane of the cell. Because the observed Raman band modulation was convoluted with silver oxide behavior from the silver nanoparticle used for surface-enhancement, we devised a thin-layer electrochemical cell with 10 μL volume using ITO and screen-printed electrodes to trap the bacterial cells without Ag nanoparticles. These experiments paved the way for studying the behavior of *M. xanthus* drop-cast on Ag-based metasurfaces. In parallel with the Raman experiments, we used epi-illumination fluorescence to image green fluorescent protein (GFP)-tagged *M. xanthus* without lysing. The underlying Ag structures produce native photoluminescence which impacts the ability to exploit hyperbolic dispersion for super-resolution fluorescence.

Next, we performed chemical imaging experiments to characterize signatures of *M. xanthus* under differing growth states. As noted above, when nutrients are scarce, *M. xanthus* exhibits multicellular fruiting bodies that harbor myxospores - enabling survival until favorable conditions return. We observed signatures of three previously unidentified secondary metabolites produced by *M. xanthus* that are attributable to the

transition from nutrient rich to starvation conditions and the development of fruiting bodies. In particular, *M. xanthus* grown on nutrient replete 1% Casitone-Tris (CTT) medium exhibit uniform expansion and yellow pigmentation, as expected. *M. xanthus* deposited on nutrient-deficient Tris-phosphate-magnesium (TPM) medium develop fruiting bodies. *M. xanthus* grown on 0.5% CTT exhibit a mixture of phenotypes where fruiting bodies form away from the edges of expanding colony growth; also, the yellow pigmentation is less pronounced compared to colonies grown on 1% CTT. Confocal Raman microscopy (CRM) imaging combined with principal component analysis (PCA) was used to characterize the spatial organization of *M. xanthus* features.^{8,10} A metabolite signature at 1559 cm⁻¹ was assigned to DKxanthene, which has previously been implicated in the yellow pigment and has also been implicated in development. Similarly, a metabolite at 1510 cm⁻¹ was assigned to lycopene, which has not previously been associated specifically with *M. xanthus* development and sporulation - a new biological discovery. The third metabolite at 1538 cm⁻¹ has yet to be identified, although we narrowed the list of possible candidates. These three metabolites also exhibit differing spatial patterning. DKxanthene signatures appear over the entire bacterial community, while signatures for the third metabolite (1538 cm⁻¹) are present only near the middle of *M. xanthus* communities. These data represent significant new molecular level understanding in the chemical signatures exhibited by the model organism, *M. xanthus* in differing environmental nutrient conditions.

Wide-field fluorescence microscopy ($\lambda_{\text{ex}} = 458 \text{ nm}$), was used to obtain images of individual *M. xanthus* cells on indium-tin-oxide (ITO) electrodes.^{6,9} Since the bacterial cells were not labeled, the origin of fluorescence was native species, likely flavoproteins. Interestingly, most cells exhibit strong, spatially-segregated fluorescence at the ends, suggesting membrane-bound flavoproteins in the ends. Moreover, dynamic imaging showed physical reconstruction of individual bacteria, *i.e.* expanding and shrinking of individual cells, likely arising from interfacial stress effects. After successfully imaging individual bacteria, we attempted direct observation of flavoprotein electrochemistry. Challenges include inhibition of electron-transfer by the cell membrane and/or interference from potential-dependent intrinsic ITO luminescence. We have recently developed a micropore electrode array for capture and trapping of individual bacterial cells which shows excellent prospects for exploring *in situ* spectroelectrochemistry of individual bacterial cells.

Finally, we utilized CRM to examine the spatiotemporal distribution of chemical signatures secreted by *M. xanthus* and their response to nutrient availability, *i.e.*, metabolic state. Ten distinct spectral features were observed by CRM from *M. xanthus* grown on nutrient-rich medium. However, when *M. xanthus* was constrained to grow under nutrient-limited conditions, it developed fruiting bodies, and the accompanying Raman microspectra were dramatically altered. Reduced metabolic states were accompanied by reduced, or completely eliminated, characteristic Raman features at 1140 cm⁻¹, 1560 cm⁻¹ and 1648 cm⁻¹. In their place, a feature at 1537 cm⁻¹ was observed, this being tentatively assigned to a transitional phase important for cellular adaptation. In addition, principal component analysis heat maps were used to illustrate how fruiting bodies in the center co-exist with motile cells at the colony edge.⁸

Products:

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Potential Benefits/Applications of DOE Funded Research: The experimental results together with numerical demonstration of super-resolution provide a pathway for super-resolution imaging of *M. xanthus* using simplified nanoridge arrays. The nanoridge geometry can be controlled to implement various super-resolution imaging paradigms.