A plasmonic imaging method for real-time spatial profiling of extracellular signaling molecules during live-cell timelapse microscopy using light microscopes

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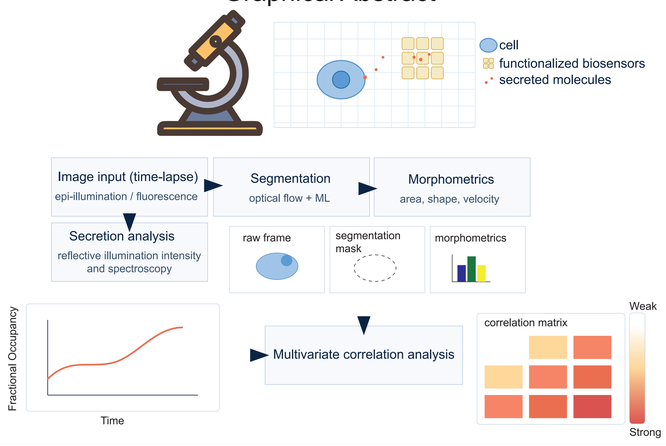
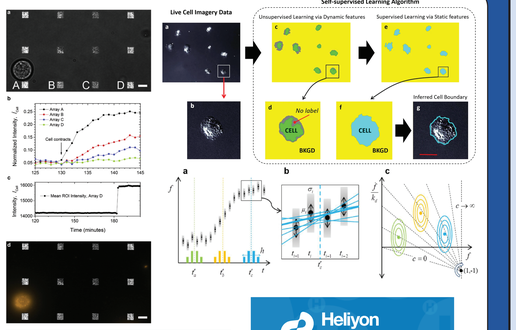
Background

Abstract: The ability to directly observe cell-secreted proteins with spatial resolution has long been a barrier in cell biology and disease research. Conventional assays such as ELISA quantify secreted factors only in bulk, obscuring heterogeneity and preventing insight into how individual cells contribute to their microenvironment. Herein we present a nanoplasmonic imaging platform that enables real-time, label-free detection of secreted proteins with spatial resolution compatible with live-cell microscopy on standard light microscopes. Our approach employs patterned nanoplasmonic sensors integrated into a microscopy-compatible substrate, allowing direct visualization of secreted molecules as they interact with the surface using light microscopes common to life science laboratories. To reduce the data processing bottleneck associated with time-lapse microscopy, we developed two automated analysis pipelines that (1) determine the fractional occupancy of the detected analyte and (2) employ optical flow computer vision with machine learning (ML) for segmentation and morphometric parameterization of cells. Combined, this plasmonic imaging hardware and dual analysis approach enables secretion-phenotype multivariate correlation. By bridging the gap between bulk secretion assays and cellular imaging, nanoplasmonic imaging offers a versatile platform for both fundamental biology and translational research, with applications in drug discovery, regenerative medicine, and precision oncology.

Graphical Abstract



Introduction: Localized plasmonic technologies are gaining increasing attention for their ability to spatially resolve molecules in solution, including proteins used in cell-to-cell communication. Localized surface plasmon resonance imaging (LSPRi) is based on the principle of light-excitable resonating electrons in metals which induce both a redshift and an increase in reflective scattering intensity when molecules bind to the metal surface. Furthermore, the technology can be integrated with other optical technologies, as long as consideration to resonance wavelengths are taken, including light microscopy. This enables researchers for the first time to witness, in a quantifiable way, cell secretions as they occur. This effectively results in temporal and spatial resolution of cell secreted molecules while providing image data on the specific cells responsible for secretion.



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Results

Fig. 1) Nanocrine plasmonic imaging chamber design. In order to achieve spatially distinct measurements from secreted proteins, in imaging chamber was utilized consisting of 2 planes separated in *z* by 100 µm. The sealed chamber uses a gas exchange-capable, optically clear surface for cell attachment to prevent hypoxia and cell death. **A)** Schematic of plasmonic imaging chamber utilizing plasmonic sensors functionalized for the detection of CSF1. **B)** Photo of plasmonic imaging chamber (top-view).

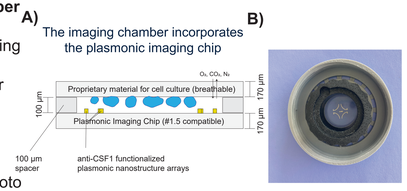


Fig. 2) Cell viability is maintained for 72 h in the plasmonic imaging chamber. To assess the impact of the chamber on cell viability, NMuMG cells (ATCC) were plated on glass coverslips followed by using no sealing material or a glass seal material or the breathable material. Cells were incubated for 72 h then stained with the LIVE/DEAD Cell Imaging Kit (ThermoFisher). **A)** Representative images of NMuMG cells 72h post treatment. **B)** Quantification of A. Original magnification: 4X. *n* = 3. ****$p \leq 0.0001$. ns = not significant.

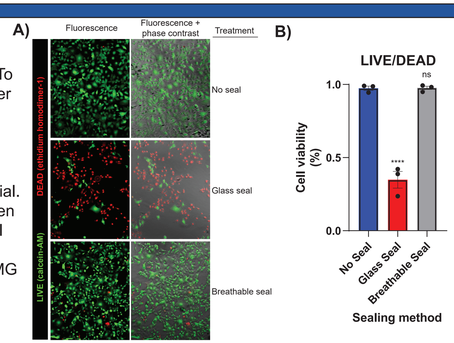
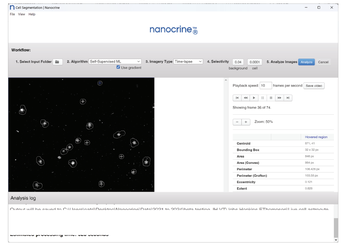


Fig. 3) Optical flow and self-supervised machine learning automated morphometric characterization of cells. Farneback Displacement optical flow is used to automate the training process which uses dynamic pixel values to label cells and background. Pixels with high optical flow are labeled as cell and low optical flow are labeled as background, while some pixels remain unlabeled. Entropy and gradient feature vectors are generated in this process which are then used to train and generate a Naïve Bayesian classifier model capable of labeling the previously unlabeled pixels from the unsupervised optical flow. **A)** GUI of OF SSML software. **B)** OF SSML source code benchmarked against the popular segmentation tool CellPose.

A) GUI for segmentation software



B) Evaluation of SSML relative to CellPose via F_1 accuracy scores.

# L Number of labels # O Number of objects in data set	10x phase / Hs27 Low contrast, heterogeneous morphologies & size, non-uniform background			10x TL / Dicty Debris, non-uniform background			10x phase / MDA-MB-231 Halo artifact, heterogeneous morphology & size, few cells			20x DIC / MDA-MB-231 Non-uniform background, texture			40x IRM / Hs27 Single cell, high contrast			100x Fluor / A549 Single cell, high contrast		
	#L	#O	F_1	#L	#O	F_1	#L	#O	F_1	#L	#O	F_1	#L	#O	F_1	#L	#O	F_1
CellPose	70k	23	0.480	70k	63	0.681	70k	7	0.201	70k	32	0.682	70k	1	0.959	70k	1	0.963
SSL	0	23	0.670	0	63	0.711	0	7	0.759	0	32	0.886	0	1	0.964	0	1	0.957

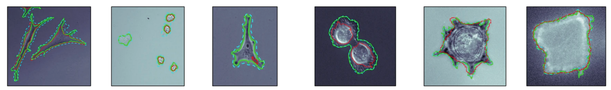


Fig. 4) Development of software for determining fractional occupancy and concentration of analyte. In order to address the bottleneck challenge for the determination of unknown concentration of analyte on a microscope, software was developed based on local linear models and temporal filtering. **A)** Software GUI that enables selection and visualization of spectra data, as well as data processing options. **B)** Software GUI that enables selection of unoccupied sensor data and maximally occupied sensor data to determine fractional occupancy over time (top). A Poisson counting process model is applied to the data to determine the fractional occupancy curve (bottom).**C)** Numerical analysis of spectroscopy data for a biotin-neutravidin binding study. A selection of 80 spectra recorded immediately after neutravidin was added to the medium, with an averaging window of 3 nm (top-left) with a magnified zoom of spectral peaks (top-right). Fractional occupancy calibration is shown with zero occupancy highlighted in blue and full occupancy highlighted in red (bottom-left). This is algorithmically computed to a fractional occupancy over the duration of the study using the maximum log-likelihood of a Poisson counting process to determine the mean intensity count at each wavelength (bottom-right). **D)** Simulated time-dependent fractional occupancy with added Gaussian noise typical of the experimental setup (left). Calculated concentration (right). The symbols and error bars represent the calculated mode of the concentration distribution divided by the KD over a 5% to 95% confidence interval.

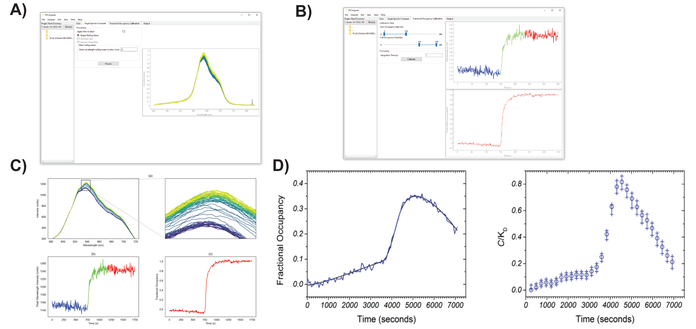
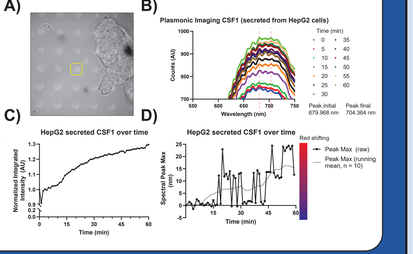


Fig. 5) CSF1 secreted by HepG2 cells is detected by plasmonic imaging. HepG2 cells were seeded onto the breathable clear substrate and incubated for 24 h. For plasmonic imaging experiments, HepG2 cells were added to the plasmonic imaging chamber after several brief washes with serum-free MEM. The imaging chamber was immediately placed on the microscope for recording spectra from a nanostructure array. **A)** Imagery showing 2 *z*-planes with the nanostructure arrays out of focus. **B)** Spectra recordings at 5-minute intervals. **C)** Quantification of intensity change in B. **D)** Characterization of spectral peak shift in B.



Conclusions and future directions

Conclusions

- A plasmonic imaging approach (sensor design, biochip design, gas permeable biochip chamber design) is established for simultaneous data capture of cell imagery and protein secretion dynamics.
- Data processing bottlenecks have been addressed through automated software approaches.
- OF SSML software outperforms CellPose and requires no training or labels.
- CSF1 secretion was quantified relative to time from living HepG2 cells; dynamic data not possible with ELISA

Future Directions

- Add local linear model function from MATLAB work to microsoft executable software for translating fractional occupancy to concentration values.
- Combine morphometric analysis with secretion data to build multivariate correlative data sets.