208. 単一細胞における細胞状態の光学的計測法の高感度化

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Introduction

Single-cell profiling methods have become more and more important with the increasing need to understand the inherent diversity in individual cells within a population. This study aims to improve our ability to study single cells in an unmodified and non-invasive manner by enhanced optical spectroscopic techniques. Current single cell methods involve a trade-off between sensitivity and invasiveness. For ultimate sensitivity, cells are broken apart and sequenced by PCR or run through analysis such as mass-spec, with detailed molecular analysis, but with destruction of the sample. Less invasive methods such as fluorescence (e.g. FACS) can elucidate the expression of certain targets such as surface receptors, without damaging the sample, but still require the addition of a label for specific targets of interest, which can inhibit the functionality that is being studied. On the other hand, completely label-free and non-invasive methods have advantages in assessing cellular composition and function without changing the cell. This project aimed to improve tools for detection of subtle cell states, and to evaluate whether nanoparticle enhancement could be a useful additional method to boost the robustness of cell profiling. Previously, we generated three different methods to try and improve the usefulness of spectroscopic cell profiling. The first was to examine individual surface-enhanced spectra within a full spectral image, and determine on a pixel by pixel basis whether each spectra contained useful information［1］. A second was to use photochemistry to create nanoparticles inside cells, which could then be exploited to read-out spectral information［2］. More recently, we implemented methods to optimize the actual readout as shown in Figure 1, using spatial averaging to improve repeatability and classification power of label-free measurements［3］. This project then used different laser methods, as shown, and evaluated the use of label-free methods for cell discrimination, classification, and dynamics over time. It also evaluated the creation of nanoparticle-enhanced substrates, particularly for challenging cell types such as lymphocytes.

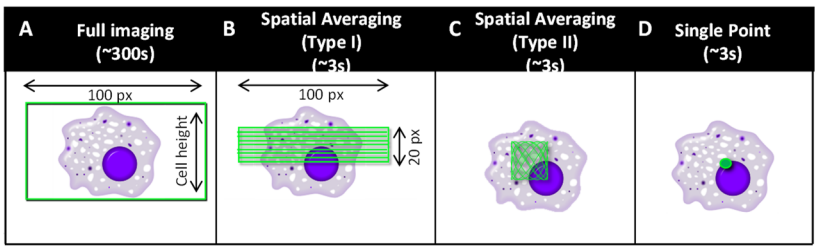

Figure 1. Different methods of applying laser exposure, throughput vs resolution
Surface-enhanced Raman spectra are usually measured as either spatially resolved spectra (A), or in colloidal solutions. However, by scanning the beam rapidly across the whole sample (B), or across a targeted region (C), a single high quality spectra (B, C) can be obtained in the same time as is taken for a single point (D).

Methods

Immortalized Mouse Embyronic Fibroblast (MEF A9) and Raw264 macrophage cells were cultured on tissue culture plates with DMEM until imaging. Lymphocyte-type EL4 cells were cultured in RPMI.

Spectral measurements were carried out on either a custom Raman setup (see reference 3 for details) at 532 nm, or a Raman-11 scanning microscope (Nanophoton) at 785 nm. Measurements were made using a range of substrates, generally measurements made at 532 nm used quartz substrates and did not use gold nanoparticle enhancement due to luminescence. For these measurements a 60x 1.27NA lens was used. Measurements made at 785 nm used quartz substrates coated with poly-l-lysine for 30 minutes, air-dried, then coated in a suspension of 50 nm AuNP particles, air-dried again, then used for experiments. Different adhesion properties were investigated, using plain uncoated substrates, PLL-coated substrates, and some custom coating agents supplied by Arakawa Industries. Finally, the combination of PLL and 50 nm AuNP was used for SERS experiments.

For laser irradiation in surface-enhanced experiments, either full imaging was performed (see Figure 1A), or a spatially averaged measurement was performed (Figure 1B), scanning the beam continuously over a target area. The power density was approximately 2mW/mm2, per line and the exposure time was either 3 seconds per line in imaging mode, or 3 seconds in total for the spatially averaged measurements. These data were then compared as raw data.

For cell classification models, a high-throughput Raman measurement mode (denoted as Type II spatial averaging in Figure 1C) was used, to rapidly characterize the molecular components of each single cell. The spatially averaged sampling region was fixed, so as not to introduce bias, however, since cell sizes are inherently diverse, the coverage per cell varied between 60% and 90% of the cell area. Data were then baseline corrected and cosmic rays removed by thresholded median filtering. Classification was then done using the R software package. A brief summary of those steps is: data were classified using a regularized logistic regression model, and uniform manifold approximation and projection (UMAP) was done for comparative analysis. FACS was also performed for comparative analysis. See［4］for further details on the models, and expanded details on these methods.

Results and Discussion

We investigated adhesion of adherent cells on different substrates. Although adhesion was not expected to influence lymphocyte type cell lines, adhesion was found to be an important factor in generation of neutrophil extracellular traps, shown in Figure 2［5］. Adhesion was also important in the successful use of nanoparticle enhancement of adherent cells. With the competing factors of nanoparticle adhesion to PLL, cell adhesion to PLL, and the aggregation or “hot spot” distribution also being critical to the degree of surface-enhanced Raman signals (SERS) observed, this report focuses on the use of non-adherent cells. The figure in the abstract shows example data from cultured MEF cells on PLL-AuNP substrates, with large differences in spectral response at nearly adjacent pixels. Further studies are underway using more uniform SERS substrates, either fabricated by our lab, or by commercial SERS substrates, with spatial averaging (Type I or II) as shown in Figure 1. We also completed label-free studies of fatty acid uptake and macrophage transformation［6］.

Cell classification models were also improved generally, and different data reduction and classification methods were compared. For example, PCA/LDA, was not found to improve on our logistic-regression based model［7］. More recent approaches such as UMAP were also implemented and found to be useful in tracking

cell populations, where the label-free data could be useful supplement or even alternative to more traditional FACS based methods. See［4］and Figure 3 for details (note that each dot represents a single cell).

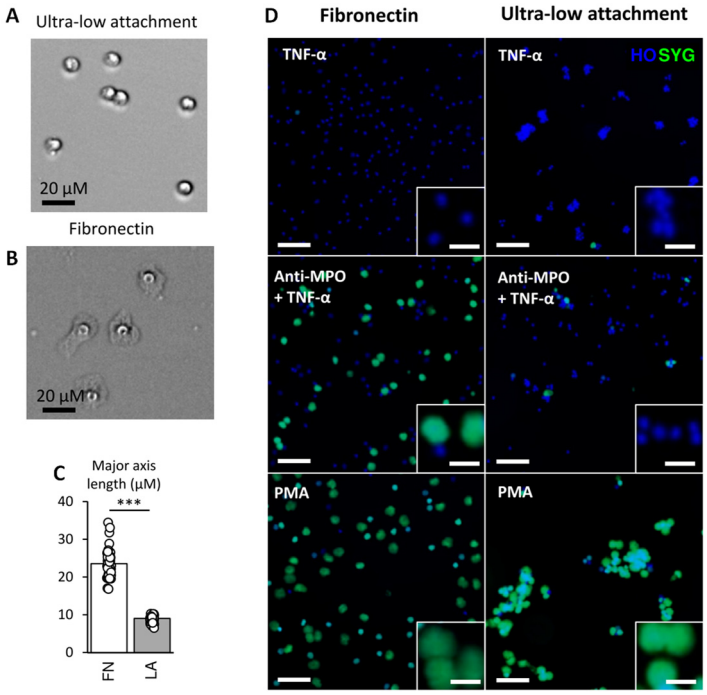

Figure 2. Substrate attachment affects neutrophil extracellular traps

On ultra-low attachement surfaces, NETs were significantly inhibited, cell morphology shown by brightfield imaging (A, B), quantified by major axis length (C), and NET response shown in green fluorescence (D). Scale bars: 100 μ m; (inset 25 μ m). See［5］for details.

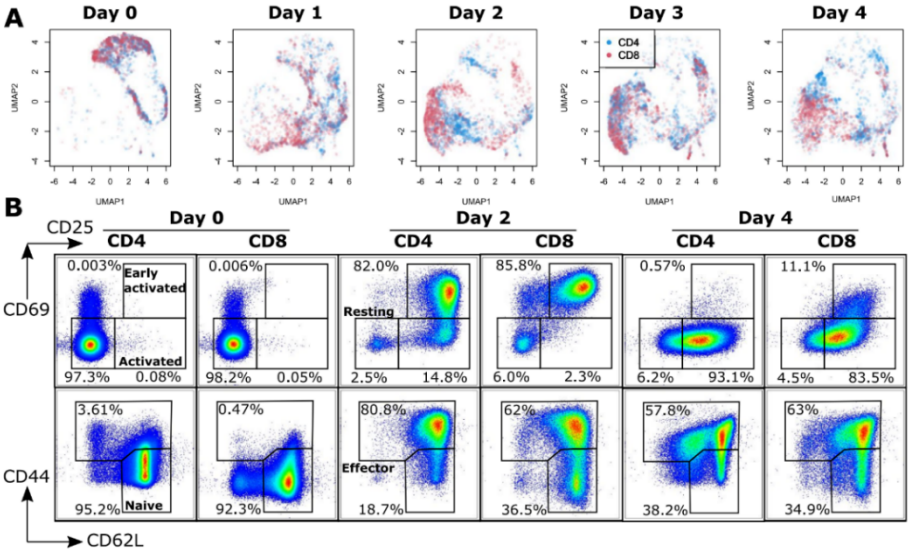

Figure 3. T cell evolution over 5 days upon activation.

(A) UMAP plots of label-free single-cell analysis data using T cells stimulated over days.

(B) for comparison parallel fluorescence-based measurements of the expression of surface markers for activation (CD25/CD69) and early differentiation (CD44/CD62L) separated per day and per cell type. See［4］for details.

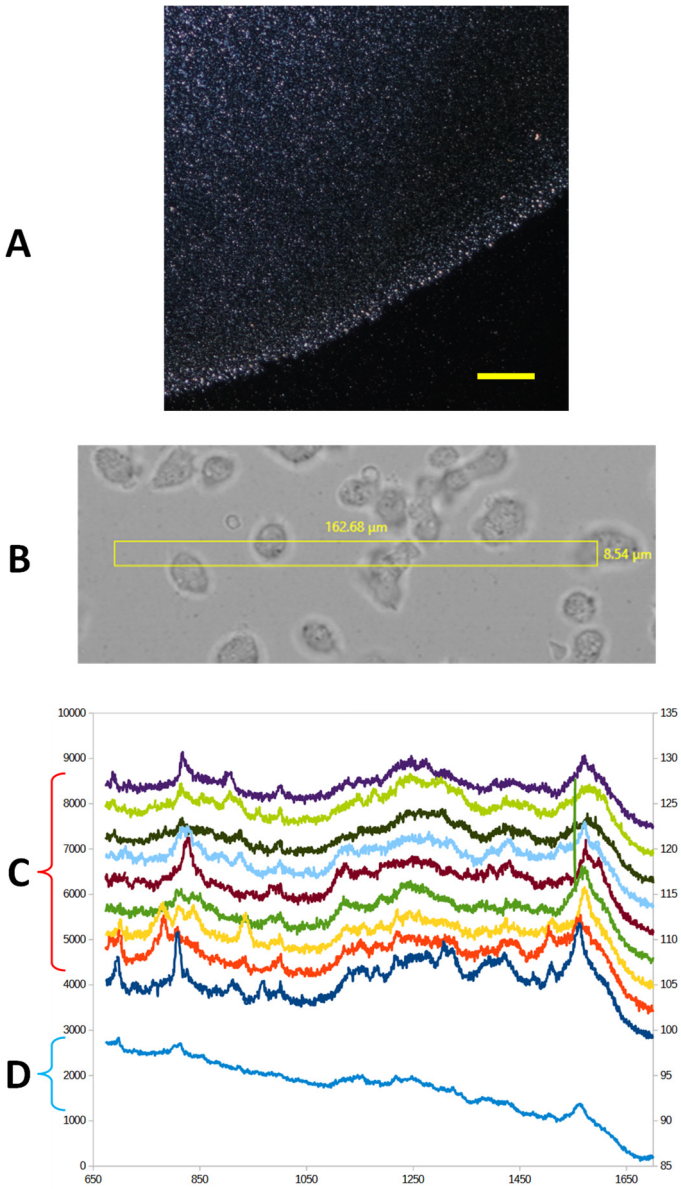

Figure 4. Beam scanning (Spatial Averaging Type I) effects on EL4 (T-cell) analysis

Top panel shows a darkfield image of the prepared substrate (A). Scale bar is 100 mm, and individual visible spots are not necessarily individual gold particles, but show the scattering from small groups of particles. (B) shows EL4 cells on the substrate, with the analysis window shown as a yellow box. Nanoparticles are present but not visible in this image. (C) shows raw spectral data quality from spatially averaged measurements (with 8 repeats), taken with 3 second exposure. (D) shows all spectral information taken in full imaging mode, averaged together, with a total exposure time of 30 seconds.

The use of spatial averaging in SERS was evaluated with promising results. In a similar manner to how it enabled high through-put Raman analysis using 532 nm [3], the nanoparticle-enhanced substrates also enabled higher throughput SERS measurements, with higher repeatability. Figure 4 shows an example region. The spatially averaged measurements used only 3 seconds, to get spectral characteristics of the EL4

T cells, while the full imaging took 30 seconds, used 10 times higher total laser exposure, and provide much worse signal-to-background ratio. Of course, while it is possible to do further selective evaluation of SERS spectra from the image data (as we demonstrated previously [1] , as a rapid evaluation, the spatially averaged approach is promising, giving high signal, high throughput, as we proposed.

Collaborators

This research was performed with collaborators in my lab, namely Dr N. Pavillon, Dr A. Hobro, and Dr P. Lelliot.

References

1) Pavillon N, Bando K, Fujita K, Smith NI. Feature-based recognition of surface-enhanced Raman spectra for biological targets. J Biophotonics. 2013 Aug;6(8):587-97. doi: 10.1002/jbio.201200181. Epub 2012 Nov 29. PMID: 23192987.
2) Smith NI, Mochizuki K, Niioka H, Ichikawa S, Pavillon N, Hobro AJ, Ando J, Fujita K, Kumagai Y. Laser-targeted photofabrication of gold nanoparticles inside cells. Nat Commun. 2014 Oct 9;5:5144. doi: 10.1038/ncomms6144. PMID: 25298313.
3) Pavillon N, Hobro AJ, Akira S, Smith NI. Noninvasive detection of macrophage activation with single-cell resolution through machine learning. Proc Natl Acad Sci U S A. 2018 Mar 20;115(12):E2676-E2685. doi: 10.1073/pnas.1711872115. Epub 2018 Mar 6. PMID: 29511099; PMCID: PMC5866539.
4) Pavillon N, Smith NI. Non-invasive monitoring of T cell differentiation through Raman spectroscopy. Sci Rep. 2023 Feb 22;13(1):3129. doi: 10.1038/s41598-023-29259-8. PMID: 36813799; PMCID: PMC9947172.
5) Lelliott PM, Nishide M, Pavillon N, Okita Y, Shibahara T, Mizuno Y, Yoshimura H, Obata S, Kumanogoh A, Smith NI. Cellular Adhesion Is a Controlling Factor in Neutrophil Extracellular Trap Formation Induced by Anti-Neutrophil Cytoplasmic Antibodies. Immunohorizons. 2022 Feb 22;6(2):170-183. doi: 10.4049/immunohorizons.2200012. PMID: 35193943.
6) Sugiyama T , Hobro AJ , Pavillon N , Umakoshi T , Verma P , Smith N . Label-free Raman mapping of saturated and unsaturated fatty acid uptake, storage, and return toward baseline levels in macrophages. Analyst. 2021 Feb 21;146(4):1268-1280. doi: 10.1039/d0an02077j. Epub 2020 Dec 21. PMID: 33346264.
7) Pavillon N, Smith NI. Deriving accurate molecular indicators of protein synthesis through Raman-based sparse classification. Analyst. 2021 Jun 7;146(11):3633-3641. doi: 10.1039/d1an00412c. Epub 2021 May 4. PMID: 33949431.