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Reagent-Free and Rapid Assessment of T Cell Activation State Using Diffraction Phase Microscopy and Deep Learning

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Abstract

CD8⁺ T cells constitute an essential compartment of the adaptive immune system. During immune responses, naïve T cells become functional, as they are primed with their cognate determinants by the antigen presenting cells. Current methods of identifying activated CD8⁺ T cells are laborious, time-consuming and expensive due to the extensive list of required reagents. Here, we demonstrate an optical imaging approach featuring quantitative phase imaging to distinguish activated CD8⁺ T cells from naïve CD8⁺ T cells in a rapid and reagent-free manner. We measured the dry mass of live cells and employed transport-based morphometry to better understand their differential morphological attributes. Our results reveal that, upon activation, the dry cell mass of T cells increases significantly in comparison to that of unstimulated cells. By employing deep learning formalism, we are able to accurately predict the population ratios of unknown mixed population based on the acquired quantitative phase images. We envision that, with further refinement, this label-free method of T cell phenotyping will lead to a rapid and cost-effective platform for assaying T cell responses to candidate antigens in the near future.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

Activated, but not naïve, CD8⁺ T cells proliferate, secrete cytokines and express numerous activation markers, which are typically assayed using flow cytometry, enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunosorbent spot (ELISpot) assay. Indeed, fluorescence microscopy, in particular, has indeed revolutionized the cellular imaging landscape by introducing targeted reporters and genetically encoded fluorescent proteins.¹ Nevertheless, the addition of dye has the following implications: (a) it perturbs the native cell structure and function; (b) dyes are susceptible to photobleaching; and (c) spectral interference due to cross-talk among multiple dye molecules adversely affect the imaging results. Alternately, vibrational spectroscopy-based imaging techniques, such as infrared (IR) and Raman microscopy, have spurred great interest owing to their ability to obtain high-fidelity molecular information without requiring any exogenous contrast agent.^{2–5} These nonperturbative techniques have been employed in various cell biology studies including mapping of the intracellular components,⁶ investigating molecular dynamics of cells undergoing mitotic division⁷ and in semiquantitative cell viability assessment.⁸ Importantly, this chemical imaging methodology has also been utilized to study blood cells including leucocytes.^{9,10} Of note, Fujita and co-workers have recently employed Raman spectroscopy to study immune cell state prediction.⁹ While this work represents an important first step toward label-free detection, its practical application is limited by the intrinsic low sensitivity of Raman scattering that constrains the sampling speed. For instance, even with a custom-built fast slit scanning Raman setup, the acquisition time for imaging a single T cell was 3 min.⁹ While it presents an intriguing set of features, spontaneous Raman imaging is not ideal for rapid cellular measurements.

Quantitative phase microscopy (QPM) is another imaging modality that does not necessitate the addition of an exogenous contrast agent for live cell imaging.¹¹ QPM, which measures

the optical path length differences, offers the quantitative assessment of morphology and nanometer scale axial motions.¹¹ Importantly, since QPM is a full field technique and entails single-shot acquisition, its imaging speed is mainly determined by the camera frame rate. While QPM has been employed to measure biophysical processes at the cellular level, most of the prior studies have been limited to understanding the morphology (volume, surface area and sphericity) and biomechanics (membrane fluctuation) of RBCs.^{12,13} Notably, erythrocyte has been principal model system due to its relatively homogeneous internal structure and surface membrane dynamics.^{12,14,15} However, recent attempts have sought to employ QPM to investigate nucleated cells and complex biological tissues.^{16–19} In particular, Park and coworkers have used three-dimensional refractive index tomography for identification of nonactivated lymphocytes.¹⁹ In a seminal study, Zangle et. al had employed phase-shifting interferometry to investigate the biomass changes in a T cell–target cell interactions.²⁰ Diffraction phase microscopy (DPM), which combines the principles of common path interferometry and single-shot phase imaging, affords high sensitivity phase measurement with unparalleled stability.^{21,22} Importantly, the DPM setup is low cost and compact,²³ and being a single-shot technique the acquisition speed is limited only by the camera speed.

Machine learning has enabled the extraction of subtle differences in images paving the way for complex image recognition and classification.^{24,25} Of note, artificial intelligence has lately been utilized in synergy with holographic microscopy for single cell studies.^{26–28} Deep learning, a subset of machine learning, is a state-of-the-art tool for generic feature extraction²⁹ and holds enormous promise for future applications in label-free cellular imaging.^{27,30,31} We decided to leverage deep learning on our phase microscopy data due to two main reasons: first, the ability to recognize innate, often latent, biological traits encoded in the images and exploit them as fingerprints; second, the capability of using raw data as inputs, thereby eliminating the need of manual feature extraction. These two attributes are crucial in view of clinically relevant applications.

Here, we have employed DPM to quantify the morphological attributes for discerning the activation state of T cells. We measured the dry cell mass from the integrated phase image acquired from viable naïve and stimulated CD8⁺ T cells. Transport-based morphometry (TBM) was harnessed to understand the subtle morphologic changes encoded in the phase images. In addition, we employed deep learning formalism to predict the population ratio of the blind mixed population of activated and naïve cells. Together, our findings reveal the enormous promise of this contrast agent-free and rapid method for identification of T cell state activation state for monitoring immune responses.

MATERIALS AND METHODS

T Cell Activation.

CD8⁺ T cells were enriched from the spleens of naïve C57BL/6 females using the Easysep Mouse CD8⁺ T cell isolation kit (Stemcell Technologies; catalog no. 19853). Enriched T cells were stimulated with magnetic beads coupled to anti-CD3 and anti-CD28 antibodies (Thermofisher; catalog no. 11456D) for 24 h. Unstimulated T cells were cultured in absence of the activating beads. All cells were cultured in RPMI supplemented with 2-

mercaptoethanol, sodium pyruvate, MEM nonessential amino acids, penicillin, streptomycin, glutamine, and 5% fetal calf serum. At the end of the incubation, stimulated cells were transferred to microcentrifuge tubes and placed on a magnet to separate the beads from cells. Both naïve and activated T cells were suspended in PBS and sent for DPM measurement. To determine the activation status, expression of CD69 by CD8⁺ T cells was tested (Figure 1). Cells were washed twice with PBS (Thermofisher; catalog no. 10010023) and stained with anti-CD3-AlexaFlour488 (Biolegend; catalog no.100210), anti-CD8-PerCP/Cy5.5 (Biolegend; catalog no. 100734), anti-CD69-PE/Cy-7 (Biolegend; catalog no. 104512) antibodies and eFluor 780 (Thermofisher; catalog no. 65-0865-14), a viability dye. Flow cytometry was performed on MACSQuant analyzer and data analyzed using FlowJo. We used three sets of known unstimulated and stimulated, obtained from three different mice. Further, eight samples with different population ratios of naïve and activated cells were generated and used to validate our findings. Experimenters performing the QPM measurements and analyses were blinded to any information on the ratios of these eight mixture samples.

QPM Measurements.

In order to acquire the quantitative phase images, we used a DPM setup, which is a part of our custom-built multimodal system consisting of an epiillumination Raman microscope system and a transmissionmode QPM system (Figure 2A), as described previously.³² We used 532 nm light obtained from a solid-state laser (Gem 532, Laser quantum) for the measurement. The light was fiber-coupled and collimated beam was used to illuminate the sample. The transmitted light was collected through a water immersion objective lens (Olympus, LUMFLN60XW, 60×/1.1). We used micropositioning stage (Optiscan 2, Prior Scientific) for mapping the sample in *X–Y* direction and a one-axis high precision translation stage to focus the objective on the sample plane. After passing through tube lens, the collimated beam was allowed to pass through the grating to generate multiple diffraction orders that are subsequently focused onto the 10 μm pinhole. Using the pinhole, we isolated the zeroth and first diffraction orders to be used as sample and reference fields, respectively. Both the beams were reflected by a mirror and focused using a lens onto a CMOS camera (Pointgrey, Flea3). The spatial overlap of these beams generates the fringes, and the interferogram thus obtained is Fourier transformed. The first order signal is chosen in Fourier space and the phase from the inverse transformed field creates the optical phase delays. In order to retrieve phase delay from the sample, we discretely measure the reference phase with no sample present in the field of view and subtract it from the phase delays for the sample. The actual magnification of the DPM system was calculated to be about 100.

For DPM measurements, 5 μL of T cell suspension was first added to a coverslip attached with secure seal spacer (Invitrogen). The sample was then sealed by placing a second cover glass onto it. The actual image acquisition time for one DPM measurement was less than 7 ms. We imaged on average 100 individual live cells for the sample sets to take intrasample variations into account. We have used three different mice to determine reproducibility of changes due to the activation process.

Transport-Based Morphometry and Machine Learning.

For our transport-based morphometry (TBM) visualization, the raw phase images were processed through Fourier transformation-based background subtraction, and then a data set was built where each figure only had one cell. The MATLAB code from a previous publication by Basu et al.³³ was used for the ensuing analysis. Briefly, the code searches a linear discriminant subspace that can present visual and quantitative phenotype variations of different cell populations through the combination between linear optimal transport and penalized linear discriminant analysis.

In order to systematically interrogate the subtle differences due to activation of CD8⁺ T cells, we have employed deep learning on the acquired DPM images. Deep learning, which is based on deep multilayered neural networks, has been employed for cell analysis including cell segmentation and classification.^{27,34} In a recent work, this approach was also successfully employed for antimicrobial susceptibility testing where live videos are used as inputs.³⁵ The deep learning model is demonstrated to discern uninhibited bacterial cells from those cells inhibited by an antibiotic without necessitating conventional image processing.³⁵ A convolution neural network (CNN) type deep learning formalism was developed and trained for this data set. By testing various different network features, a 15-layer, comprised of 1 image input layer, 3 convolution 2D layers, 3 batch normalization, 3 ReLU, 2 2D max pooling, 1 fully connected, 1 softmax, and 1 classification layer. Network specifications include a 30 training epochs, and 15 iteration validation frequencies. The training data set, used to develop the neural network, was constituted by 6 samples that had 3 fully naïve and 3 enriched stimulated cells, respectively. The independent test data set consisted of 8 samples with different mixtures of naïve and stimulated cells; these ratios were unknown to the CNN model and provided an ideal cohort for assessing the accuracy of identifying the T-cell state activation.

RESULTS AND DISCUSSION

The flow cytometry results are shown in Figure 1. The enriched population for the nonactivated cells showed a viability of 97% (top row, second panel) and 92.5% of the cells were CD8⁺ T cells (top row, third panel). T cells are difficult to culture in absence of any stimulus and the viability of culture was poor in absence of antibody-coupled beads (second row, second panel). To avoid working with poorly viable cultures, the “light experiment” was performed using CD8⁺ T cells freshly enriched from mice (first row), and cells were stimulated for 24 h with antibody-coupled beads (last row). As expected, CD8⁺ T cells cultured in the absence of the beads did not express surface antigen CD69 (second row, last panel). Stimulation of T cells with antibodies against CD3 and CD28 elicited expression of CD69 in about 71% of the cultured CD8⁺ T cell (last row, last panel).

As detailed in the Materials and Methods, we used our custom-built DPM setup for quantitative phase measurements (the schematic of the system is provided in Figure 2A). The representative phase images of naïve and stimulated T cells are shown in Figure 2B and C, respectively. We reasoned that morphology, being an important functional characteristic in cell phenotyping,^{36,37} would allow us to discern the cell activation state. In particular, cell mass has previously been utilized for various purposes including phenotyping and

elucidation of growth dynamics.^{38,39} Here, the (nonaqueous content) dry mass of a live cell was calculated from surface integral of the optical phase shift, as detailed in earlier reports.^{38,40,41} The calculated dry masses of naïve and activated T cells are presented in Figure 3. As shown, the average dry mass of activated cells is significantly higher in relation to that of the naïve cells (p -value < 0.001). It is worth noting that we measured on average 100 individual live cells from the mouse samples to obtain a robust data set. Further, we recorded these phase images from three different mice, which reinforced the reliability of our previous observation. While the dry masses of naïve cells are relatively tightly clustered, the dry cell masses of activated cells exhibit more spread. This may be attributed to the differential activation and the difference in the time period between PBS washing and DPM measurement. The average dry mass calculated from all three measurements for naïve and activated cells were 33.1 ± 13.1 and 53.8 ± 32.4 picograms, respectively. The dry mass values of the naïve cell population are comparable to the observations of an earlier report using 3D tomography that focused on CD8⁺ naïve cell.¹⁷

After measuring the cell masses of known naïve and activated populations, we performed phase measurements on blind samples consisting of only naïve, only activated and mixed populations in different ratios. Prior to measurements, these samples were randomly labeled to avoid any clue for sample identification. From each sample, around 100 different random live T cells were subjected to phase imaging measurements. The average dry masses of the blind samples are presented in Figure 4A. As evident from the figure, sample nos. 1, 6, and 8 show a similar dry mass value and also tight data clustering. Based on earlier data in Figure 3, one can reasonably infer that the dry cell mass of this sample group is similar to that of the naïve cells.

On cross-verification with the immunologists (S.H.K. and P.K.S.), this was found to be consistent with the ratios of the naïve and activated cells used to create these test mixture samples. Information on the ratios is provided here in Figure 4B. It is worth noting that, among the three samples (#1, 6, and 8), sample #8, which has a relatively higher content of activated cells, also has a larger number of outliers in the dry cell mass data. Of the remaining samples, sample #2 (that has an equal proportion of naïve and activated T cells) displays relatively lower dry cell mass compared to the rest. It is also notable that a larger spread is observed in the dry cell mass values of the mixture samples with a high proportion of activated cells.

Moreover, we employed TBM on the phase images to identify the morphological mode(s) of maximum variance in the naïve and activated cells. The information revealed using this methodology is presented in Figure 5A and reveals the subtle, but visually discernible, differences between the naïve and activated cell distributions. Here, the horizontal axis, with unit of the standard deviation, illustrates the typical images corresponding to each histogram coordinate along the most morphologically discriminant direction between the naïve and activated cells. The associated p -value of histogram separation computed using the Kolmogorov–Smirnov test is less than 0.001, suggesting that the differentiation between the naïve and activated subpopulations is highly statistically significant. We, subsequently, studied the relationship between the corresponding dry mass values and the projection scores of the most discriminant direction as identified by the TBM algorithm. As shown in Figure

5B, a strong linear correlation ($R^2 = 0.97$) is observed between these two parameters, indicating that the major morphological difference between the two cell classes stems from the variations in cell size.

While the cell dry mass appears to be a crucial indicator of the cell activation state, it is challenging to predict the actual population ratio based on this single parameter. To address this limitation, we designed a deep learning framework to quantify the unknown population ratio on eight test samples. For the training, we used approximately 300 phase images each for naïve and activated category obtained from three different mice. The schematic of the deep learning formalism is given in Figure 6A. Further details of the layered architecture are provided in the Materials and Methods. We selected deep neural networks as the statistical model to learn from the data set as they can express many patterns and lead to systems with superhuman performance.⁴² Here, we used the acquired phase images as input for machine-learning algorithm development. Figure 6B provides the results of the prediction on the test data alongside the actual ratios of the known and activated cells in these eight samples. The true and predicted values of the mixture ratios are very close (and are nearly identical for some of the test samples). The discrepancies in the predictions may be attributed to our underlying assumption that the cells in the known population are either 100% naïve or 100% activated. While this may be true for the naïve cells, it is nearly impossible to ensure that the cells in the activated sample were fully stimulated, without use of any exogenous contrast agent, leading to the prediction errors on the test data. This result corroborates the predictions based on flow cytometry and demonstrates the utility of the deep neural network for biologically meaningful cell state identification based on quantitative phase images. Toward the ultimate goal of advancing these preliminary findings for routine measurements of clinical samples, there are two major focal points of our future investigations. First, expanding the sample set will allow us to establish a more robust and accurate deep learning classifier and, simultaneously, to probe further as to which key biological traits are responsible for identifying activation of the T cells. Second, an implicit assumption in our current study surrounds the percentage of stimulated cells in the activated samples. Using fluorescence-activated cell sorting (FACS) to establish the ground truth on a single cell basis, we will refine our deep learning model to predict activation state without the aid of the former assumption.

CONCLUSION

In this study, we have demonstrated that DPM has the capability to discern real-time morphological changes associated with T cell activation, without necessitating the addition of any contrast agents. Specifically, QPM-based measurements were used to identify the activation state of T cell, which points toward its promise as potential reagent-free and low-cost tool in immunology. Our results show that the average dry mass of activated T cells is significantly higher as compared to that of the naïve cell, and the increase in mass change is strongly correlated with the cell size. Along these lines, we showed that morphological changes emanating from activation could be harnessed to construct decision algorithms with high diagnostic power. The proof-of-concept deep learning network, developed here, could also continue to improve its performance with additional training examples. Future studies exploring the change in other morphologic attributes of naïve T cells upon stimulation are

important for a comprehensive understanding of the activation process and their expanded use as diagnostic and prognostic markers in immunology. When compared with the other label-free technique such as Raman spectroscopy, the proposed DPM approach for the identification of CD8⁺ cell activation stage is faster by several orders of magnitude. We anticipate that, by leveraging the wealth of morphologic information encoded in the QPM data, one can not only extend this method for phenotype profiling of other cells but also use it to recognize latent morphological modifications in response to stimuli.

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Figure 1.

(A) CD8⁺ T cells enriched from a naïve mouse;

(B) CD8⁺ T cells enriched from a naïve mouse and cultured for 24 h without stimulus;

(C) CD8⁺ T cells enriched from a naïve mouse and stimulated for 24 h with anti-CD3/CD28 beads. FSC: forward scatter; SSC: side scatter.

Figure 2.

(A) Schematic diagram of the QPM system used: M, mirror; TL, tube lens; Representative phase image of (B) naïve and (C) activated cell live cells. The color bars represent phase values with a unit in radian. The scale bars are 10 μm .

Figure 3. Scatter plots of dry cell masses for three independent naïve and activated samples: blue, naïve; orange, activated. Each symbol represents a single-cell measurement. Horizontal black lines: mean value; vertical lines: standard deviation; *** $P < 0.001$.

Figure 4.

(A) Average dry mass of the blind samples with different population ratios. Each data point corresponds to the dry mass of an individual live cell computed based on the acquired phase image.

(B) Known population ratio of the samples.

Figure 5.

(A) Histograms of the projections of the coordinates of images of the naïve and activated T cells on the most discriminant direction. The Kolmogorov–Smirnov p-value was computed as 0.001;

(B) Plot showing the correlation between dry mass and projection score of the most discriminant direction. R^2 value was determined to be 0.97.

Figure 6.

(A) Schematic of deep learning formalism used in this study. CN = convolution layer, BN = batch normalization, RELU = rectified linear units, PL = max pooling layer, FC = fully connected layer, SM = SoftMax layer;

(B) Deep learning-based predictions of the composition of the test samples. The actual ratios of the naive and activated cells are provided alongside.