QUANTITATIVE ANALYSIS OF LYMPHOCYTE MEMBRANE PROTEIN REDISTRIBUTION FROM FLUORESCENCE MICROSCOPY

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ABSTRACT

The relocalization of plasma membrane proteins is critical for establishing cellular polarity and regulating cell signaling. Three-dimensional fluorescence video microscopy allows the dynamic visualization of proteins in living cells. We have developed a robust and automated method to employ fluorescence data acquired in this manner for quantitative analysis of membrane protein movements across the cell surface. Our method utilizes level-set-based surface reconstruction followed by a maximum likelihood surface registration algorithm for rigid-body alignment of noisy images. A surface-walking technique yields distance maps for the cell surface, which are then used to measure changes in protein surface distribution over time. Applying this method to signaling in T lymphocytes, we have used it to monitor receptor movements and have validated these results against previously reported single-particle tracking data.

1. INTRODUCTION

Relocalization and clustering of signaling proteins in the plasma membrane drive a diverse array of biological processes including lymphocyte activation, neuronal synapse formation, apoptosis, and cell motility. In all of these cases, redistribution of membrane-associated signaling proteins has been associated with the initiation of cellular signaling cascades and the establishment of cellular polarity. Protein localization is commonly monitored using fluorescent probes, which can be visualized in living cells using four-dimensional (x,y,z,t) microscopy. Patterns of protein localization yield information about the regulation of these signaling processes and their failure in disease. Quantitative methods for protein relocalization analysis are thus needed to assess these patterns systematically and to enable mechanistic analysis of the underlying signaling networks.

T lymphocyte activation provides a well-studied example of regulation via membrane protein localization. During stimulation with antigen, a number of signaling and adhesion proteins cluster at the interface between the T cell and the antigen-presenting cell in an organized fashion. Among the proteins that cluster at this interface are the T-cell receptor, its ligand the major histocompatibility complex protein, and the T-cell-receptor-associated signaling protein CD3ζ. Despite extensive qualitative study, the precise functions of these phenomena have yet to be determined, and more quantitative data will assist in the development and testing of models for signaling mechanisms.

Previous work on analysis of protein distributions has included 2-dimensional analyses and those based on spherical models [1, 2]. Three-dimensional work has been performed on a related problem of object tracking (labeled spots corresponding to either single or multiple particles) [3, 4], although these have not explicitly included surface analyses. Recently, level-set-based approaches have been used to measure organelle volume changes [5, 6]. Our method utilizes a model-free surface-based approach to measure changes in protein distribution. This allows the capture of global as well as local distribution changes and increases the accuracy of measurements on often-irregular cellular surfaces.

Our approach utilizes a segmentation filter previously reported [2] for identification of membrane voxels. We subsequently perform level-set surface reconstruction at each time point [7] and surface registration using a maximum likelihood approach. Identification of a biologically relevant origin for distance measurement is performed via clustering analysis. A graph labeling algorithm is then applied to calculate the shortest surface distance from the origin to each surface point. Combination of this distance information with membrane voxel intensities yields a time series of distance-intensity distributions. In this report, we perform this analysis on clustering of T-cell-receptor-associated CD3ζ molecules and validate the results with reference to T-cell-receptor single-particle tracking data.

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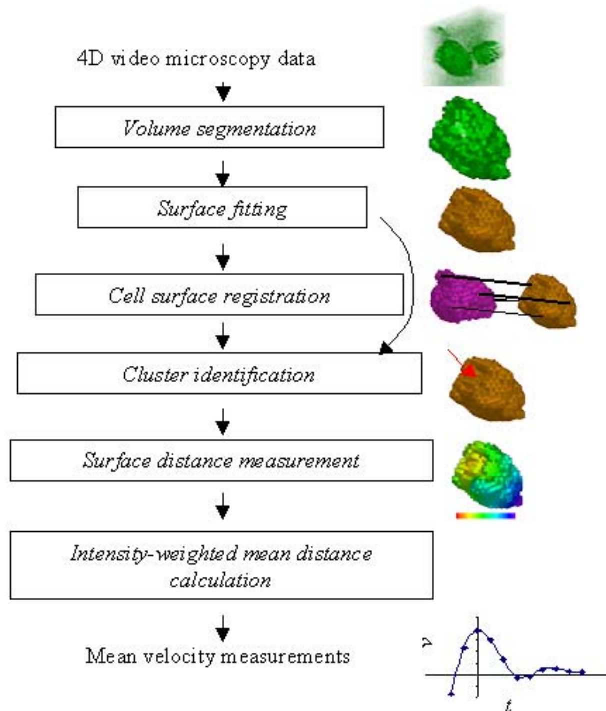

Figure 1. Outline of the analytic process

Shown are the sequential stages in our analytic system accompanied by volume renderings from progressive analytic stages of a single time point from a CD3ζ-GFP dataset.

2. SURFACE RECONSTRUCTION AND REGISTRATION

Our segmentation filter, used courtesy of William Moss [2], offers increased specificity for membrane structures when compared to a simple edge-detection filter. For accurate distance mapping, however, we desire a smooth, continuous surface corresponding to the plasma membrane geometry at each time point. To that end, we perform level-set-based surface reconstruction as described by Zhao et al. [7] using the membrane points defined by our segmentation filter as the target dataset.

We then align the surface thus derived at each time point against each adjacent time point using a pairwise registration scheme. We consider rigid-body transformations and perform a global optimization technique across the 7-dimensional quaternion rotation and translation space using iterative line searches with descending step size. Our search metric is a maximum likelihood criterion: p (D | M,T), where D is the data image, M is the model image, and T is the transform.

$$p(D \mid M, T) = p(T * D \mid M)$$

By conditional probability,

$$p(T * D \mid M) = \frac{P((T * D) \cap M)}{P(M)}$$

$$p((T * D) \cap M) = \frac{\sum_{\text{voxels x}} (\mathrm{T} * \mathrm{D})_x \text{ AND } M_x}{\sum_{\text{voxels x}} (\mathrm{T} * \mathrm{D})_x \text{ OR } \mathrm{M}_x}$$

We then maximize this probability across transform space as described above. Using this process, we obtain a set of rigid-body transformations describing the motion of the cell membrane over the period of observation.

3. CLUSTERING ANALYSIS

For surfaces isomorphic to a sphere, a continuous two-dimensional parameterization that preserves surface distance is impossible. We therefore identify the biologically relevant reference point for distance measurement (here the center of the cell-cell interface where receptor clustering is occurring) prior to parameterization. We then perform a one-dimensional surface parameterization to yield a radial distance-intensity distribution for each time point.

Because the interface between cells under consideration develops very dynamically, often changing with respect to cell shape, accurate identification of the interface center, and thus the reference point for parameterization, is challenging. We determine the center based on k-nearest-neighbors clustering analysis at a single time point. For each point, we find the 15 nearest neighbors and assign a clustering metric of $\sum_{i \in \text{neighbors of point p}} \text{intensity}(i) G(d_p(i))$, where $d_p(i)$ is the surface distance from p to i and G is a Parzen window function, in this case a Gaussian with σ=2.5 mm. The surface point with the maximal clustering metric is designated the reference point.

The surface registration information obtained above is then used to map the reference point to all time points. We do not repeat the clustering analysis at each time point because such consideration would bias subsequent assessment of clustering behavior. However, our analyses have shown that the use of intensity data for clustering analysis at a single time point does not impair our ability to measure clustering phenomena in our lymphocyte activation system (data not shown).

4. DISTANCE MEASUREMENT AND VELOCITY ANALYSIS

We perform surface distance parameterization using a graph labeling strategy for surface walking based on an upwinding scheme. This algorithm will visit all connected nodes on the surface and will label them with a good approximation of the surface distance. Radial distance-intensity distributions are then obtained by mapping the surface distance information to the original membrane points identified by the segmentation filter and defining the fractional intensity F at each distance x and time t to be $F(x,t) = \frac{\sum_{p,d(p)=x} Intensity(p,t)}{\sum_{\forall p} Intensity(p,t)}$ for membrane points p. The mean intensity-weighted distance at time t is thus $\overline{d}(t) = \sum_{\forall x} F(x,t) \cdot x$. We assess mean velocity via two measures: $\Delta x/\Delta t$ and a 5-point linear fit that has lower noise but is temporally smoothed.

5. EXPERIMENTAL RESULTS

Using the method described above, we have analyzed receptor movements during T lymphocyte stimulation. Cell culture and microscopy were performed as previously described [8, 9]. The fluorescently labeled protein under study was CD3-ζ, a membrane signaling protein associated with the T-cell receptor. Representative distance-intensity

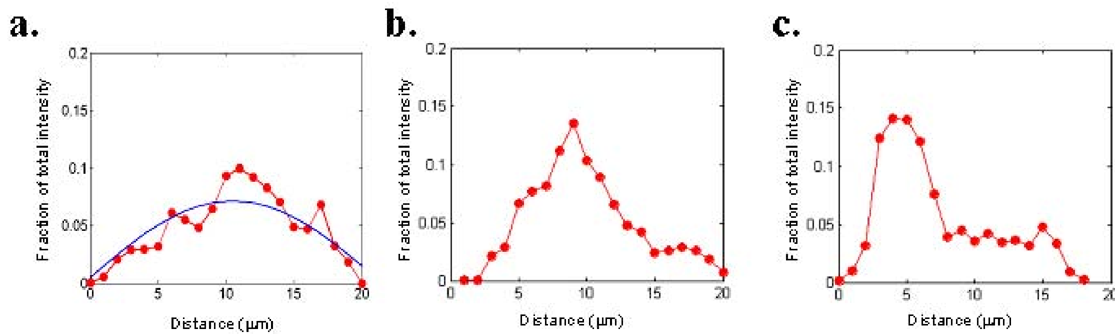

Figure 2: Radial distribution of intensity.
Plotted are distributions showing the fraction of total intensity present at each surface distance increment from the reference point. The distribution plotted in a. is at 2.5 minutes prior to activation, the distribution in b. is at the time of activation, and the distribution in c. is 3.5 minutes after activation. Mean intensity-distance values are 10.9, 9.9, and 6.7 µm respectively. The blue line shows for reference a distribution corresponding to uniform intensity across the surface of a sphere.

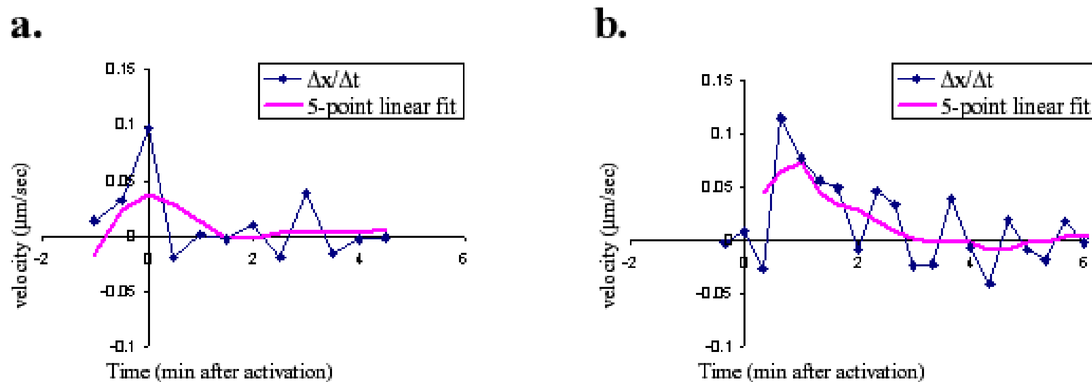

Figure 3: CD3zeta receptor velocity in response to T lymphocyte stimulation.
a. shows velocity measurement derived from bulk fluorescence measurements via our method. b. shows a single-particle trace from the published data [1]. We have calculated velocity for each trace both using the $\Delta x/\Delta t$ velocity determination method and using 5-point moving window linear fits.

distributions are shown in Figure 1, and a trace corresponding to the mean velocity of all receptors across the surface of a single cell is plotted in Figure 2. As shown in these figures, our data confirm the qualitatively observed receptor clustering behavior upon T-cell activation. To further validate our method, we compared our mean velocity measurements to previously reported single-particle tracking data for T-cell receptor movements (Figure 2). CD3-ζ motions are thought to approximate those of T-cell receptor, a hypothesis consistent with our observations. We measure a peak receptor velocity within 16% of the single-particle tracking data. The single-particle trace shows a slower decay in the initial velocity spike than our mean velocity observations, but the overall behavior is quite consistent. As our measurements represent a population mean, it is expected that some of the receptors will have a more extended velocity profile, particularly those that start farther away from the cluster center and subsequently incorporate. The minor discrepancies between our observations and those from single-particle tracking thus illustrate how analyses of bulk movements provide complementary information to single-particle tracking experiments. Bulk analyses can be performed on a broader range of experimental systems and yield population statistics much more readily than laborious single-particle analyses, although the latter provide a "gold standard" for movements of individual molecules.

6. CONCLUSIONS

We have presented a novel system for measurement of membrane protein movements on the cell surface. Our methods are fully three-dimensional and combine a level-set surface fitting technique with maximum likelihood-based surface registration. Applied to membrane receptor signaling during T lymphocyte activation, this analytic system measures mean CD3-ζ velocities, yielding results that are consistent with previously reported single-particle tracking experiments. Since our analytic framework is generally applicable to membrane protein movements, we anticipate it to be of use to investigations of cell signaling in other systems such as neuronal synapse formation and cellular direction sensing.

7. REFERENCES

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