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# Automated line scan analysis to quantify biosensor activity at the cell edge

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ABSTRACT

Biosensors are valuable tools used to image the subcellular localization and kinetics of protein activity in living cells. Signaling at the edge of motile cells that regulates cell protrusion and retraction is important in many aspects of cell physiology, and frequently studied using biosensors. However, quantitation and interpretation is limited by the heterogeneity of this signaling behavior; automated analytical approaches are required to systematically extract large data sets from biosensor studies for statistical analysis. Here we describe an automated analysis to relate the velocity at specific points along the cell edge with biosensor activity in adjoining regions. Time series of biosensor images are processed to interpolate a smooth edge of the cell at each time point. Profiles of biosensor activity ('line scans') are then calculated along lines perpendicular to the cell edge. An energy minimization method is used to calculate a velocity associated with each line scan. Sorting line scans by the proximal velocity has generated novel biological insights, as exemplified by analysis of the Src merobody biosensor. With the large data sets that can be generated automatically by this program, conclusions can be drawn that are not apparent from qualitative or 'manual' quantitative techniques. Our 'LineScan' software includes a graphical user interface (GUI) to facilitate application in other studies. It is available at hahnlab.com and is exemplified here in a study using the RhoC FLARE biosensor.



## 1. Introduction and overview

To understand cell signaling, detailed information regarding the spatio-temporal dynamics of protein activity is required. This is quantified in living cells using fluorescent biosensors, which report the localization and kinetics of protein structural changes, including conformational change, phosphorylation, cleavage etc. [1]. Biosensors can be based on different designs and use different mechanisms to generate fluorescence (e.g., FRET, solvatochromic dyes, fluorophore–quencher interactions etc.) but, in general, biosensor studies map the distribution of protein activity across the cell; each pixel in an image is associated with a specific value reporting activation at that pixel. Such maps are obtained at multiple time points to reveal the kinetics of activation. These values are related to the actual level of protein activity, or the activity per molecule (i.e., the percent of molecules in the active state within a given pixel), as in the single chain ratiometric sensor of GTPase activity used here.

In motility, tight regulation of multiple processes at different regions of the cell edge generate coordinated extension, retraction, and directed movement in response to a variety of directional cues [2–4]. For example, it has been shown that Rho GTPases regulate cell edge dynamics differently at the front and rear of migrating cells [3,5–7], and that these proteins' distance from the edge determines their role in different aspects of actin and adhesion regulation [4]. Biosensors are particularly useful for quantifying the regulation of cell migration because they can dynamically assay the relationship between protein activity and local behavior of the cell edge. However, a significant obstacle to experimental interpretation of biosensor data from motile cells is the heterogeneity of cell behavior, arising from both cell-to-cell heterogeneity and variation in a given protein's behavior within each cell. The majority of this variability is inherent to the biology and can arise from local conditions, cell-cycle state, and cell morphology. How inter-cell heterogeneity arises, and how regulation is managed on a background of such variability, is an open question.

In a previous publication we applied a new tool that automatically quantifies the relationship between line profiles of protein activity and nearby edge velocity. This approach led to novel biological insights, demonstrating relationships between velocity and Src activity [8]. Here we describe this tool and its development

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in detail, and provide a user-friendly graphical user interface (GUI) to aid in its application by other researchers.

Due to cellular heterogeneity, qualitative observations based on a small number of cells or line scans are difficult to interpret, and are subject to selection bias. To limit the amount of data to something that can be handled 'manually', observers have restricted observations to subcellular regions that exemplify specific behaviors. The automated tool supplied here is designed to analyze all frames in a movie, and therefore does not introduce human bias in selectivity. It can readily provide sufficient data for statistical analysis, revealing relationships between protein activity and velocity that are not obvious to the eye, as exemplified by our previous study of Src activation [8].

Line scans are a popular way of quantifying biosensor activity at the edge of cells, where biosensor activity is recorded as a function of distance from the cell edge. Popular imaging software (for example **MetaMorph**® or ImageJ) will perform this calculation for user-defined (manual) lines. The tool LineScan described here automatically places the lines normal to the cell edge, and couples the line scan with the proximal velocity of the edge. LineScan is written in MATLAB and contains a user friendly GUI. The software package is available at hahnlab.com and http://www.med.unc.edu/pharm/elstonlab/.

Our method is complementary to the approach of Machacek and Danuser [9], which was used to provide detailed insights into spatio-temporal dynamics of RhoGTPases at the edge of migrating cells [4]. Our method, which relies on line scans as opposed to grid generation near the cell edge, can be simpler to implement in some cases, but lacks the sophisticated two-time correlation capabilities of the Machacek and Danuser approach. For thorough literature reviews and further insights into the critical necessity for automated tools in image analysis the reader is referred to Dehmelt and Bastiaens [10] and Danuser [11].

The tool presented here enables automated collection of large amounts of image data coupled to a phenotypic measure of cell behavior (edge velocity). We automate and extend 'line scan' analysis, a popular method for analyzing biosensor data. Although manual generation of line scans has been sufficient in many studies [12–15], automating the process allows large amounts of data to be analyzed, enabling new biological insights to be gained even in the presence of heterogeneity and relatively poor signal/noise ratio.

Automated line scan analysis presents several computational challenges: line scans of a fixed length may extend outside the cell interior, a direction perpendicular to the local curvature of the edge needs to be automatically established (on a scale greater than the pixel size), and it is critical to store and manipulate large amounts of data efficiently. Our approaches to these concerns are outlined below.

For novel biological insights it is essential to couple line scan measurements to a relevant phenotypic read-out. In cell migration, the velocity of the cell edge is one such read-out. Our software package calculates the edge velocity and then groups line scans in terms of this velocity, allowing signaling at different distances from the cell edge to be correlated with edge velocity. This is relevant to motility because proteins are known to have different functions in zones at successive distances from the cell edge.

## 2. Method

The algorithm for LineScan is outlined in Fig. 1. The software was developed in Mathworks' MATLAB and requires the image processing toolbox.

The starting point (Fig. 1(1)) is a movie composed of biosensor activity images. Pre-processing of raw images is required to produce biosensor activity maps. This is beyond the scope of this article, but has been well addressed in several recent methods papers [16–19]. Depending on the biosensor and imaging method used, this pre-processing can include background subtraction, shade correction, thresholding, ratio imaging, and correction for photobleaching or signal bleed through.

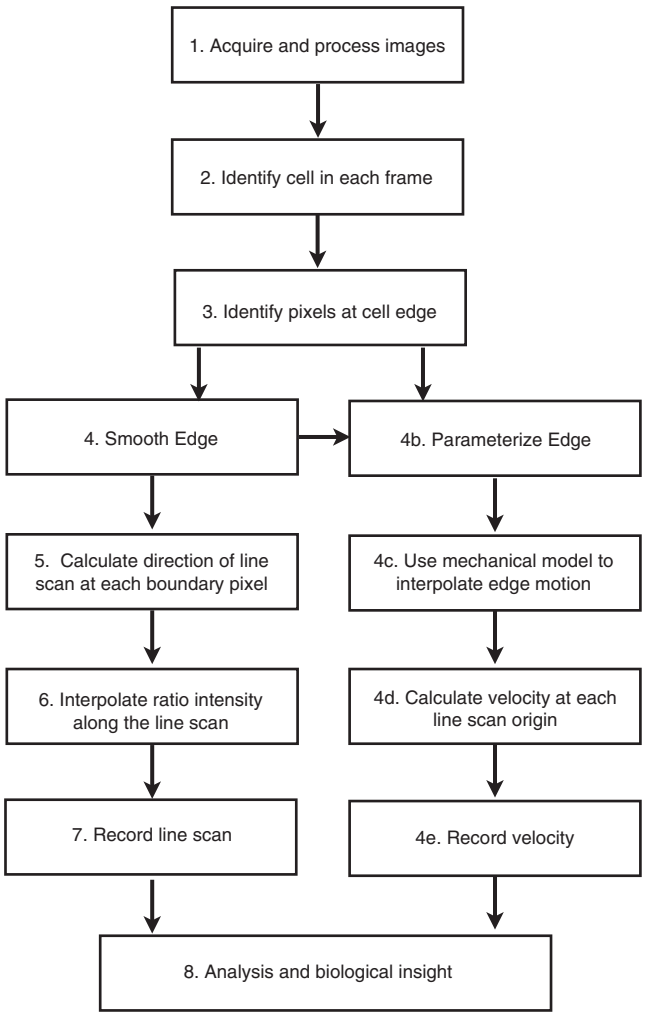


**Fig. 1.** Outline of algorithm for automatically calculating line scans and edge velocity. Line scans are automatically quantified by an algorithm, which finds the cell, finds the edge of the cell, and calculates the direction and origin of line scans for quantification. A smoothed edge is used to interpolate the velocity in a sequence of image frames (movie). Edge velocity and line scans can be calculated independent of one another (line scans steps 4–7, edge velocity steps 4b–e).

For the procedure described here, the next step (Fig. 1(2)) is to identify the cell in each frame. A well-processed biosensor image will already be thresholded such that regions of intensity below the threshold are determined to be extra-cellular or too noisy for analysis, and so are set to zero. For our purposes, identifying intracellular regions is therefore trivial. The software identifies the cell for analysis as the largest connected region in the image that is above the threshold. This excludes fragments in the image from any further processing and analysis. If two cells are in an image the software will analyze the largest. When analyzing a movie with multiple cells in the image field, care must be taken to exclude cases where different cells become the largest in each frame during the course of the movie. We recommend cropping out unneeded cells before the analysis at the pre-processing step.

To identify the pixels at the cell edge the Matlab function 'bwboundaries' is used. This will find the exterior pixels of connected regions in binary images (in this case, the binary image is