Appendix II. Quantitative immunofluorescence for measuring spatial compartmentation of covalently-modified signaling proteins

Abstract

Intracellular signaling pathways control cell behaviors and multicellular morphodynamics. A quantitative understanding of these pathways will provide design principles for tuning these signals in order to engineer cell behaviors and tissue morphology. The transmission of information in signaling pathways involves both site-specific covalent modifications and spatial localization of signaling proteins. Here, we describe an algorithm for quantifying the spatial localization of covalently-modified signaling proteins from images acquired by immunofluorescence (IF) staining. As a case study, we apply the method to quantify the amount of dually phosphorylated ERK in the nucleus. The algorithm presented here provides a general schematic that can be modified and applied more broadly to quantify the spatial compartmentation of other covalently-modified signaling proteins.

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Introduction

Signal transduction networks control all aspects of cell behavior, such as metabolism, proliferation, migration, and differentiation (1). Thus, engineering cell behaviors will hinge on understanding and tuning information flow in these signaling pathways. Intracellular signals transmit information in at least two major ways. First, signaling proteins undergo covalent modifications that alter their intrinsic enzymatic activity and/or their interactions with binding partners. In addition to the connectivity of the signal transduction network, signaling proteins are localized spatially. Where a signal is located can influence its accessibility to upstream and downstream factors, and therefore, can play a significant role in controlling information flux (2).

Green fluorescent protein (GFP) has provided a powerful way to track the localization of signaling proteins (3). Variants of GFP spanning a wide range of spectral properties have opened the door to monitoring co-localization of signaling proteins. A key challenge, however, is that quantifying signal propagation must involve not only tracking protein localization, but also the covalent state of that signal.

Sensor platforms that track both spatial localization and covalent state/activity are emerging. Several involve fluorescence resonance energy transfer (FRET), a phenomenon wherein the close proximity of two complementary fluorphores allows one (the donor) to excite the other (acceptor) (4). The quenching of the donor and the excitation of the acceptor serves as a FRET signal. One general strategy has been to

introduce a chimeric version of the signaling protein. Both the acceptor and donor are placed in the protein whose folding into an active confirmation changes the FRET signal. Examples include the Raichu sensors for the cdc42/Rac/Rho family of GTPases (5). In another design, the fluorophores have been placed in chimeric pseudosubstrates for tyrosine kinases (6)and caspases (7). When these signaling enzymes act on the substrate, the re-folding or the cleavage of the substrate changes the FRET signal. A final approach is to place one fluorophore on the signaling enzyme and the other fluorophore on a binding partner. When these are recruited to each other, FRET signal ensues. Examples of this third approach include the Raichu-CRIB sensors for Rho family of GTPases (8).

A major drawback of these tools, however, is that they are highly tailor-made and do not report on the remarkable diversity of covalent modifications that a single signaling protein undergoes. For example, the PDGF receptor is phosphorylated at multiple tyrosine residues, and each phosphorylation site enables its interaction with distinct downstream targets (9). Such multisite covalent modifications are prevalent across signaling proteins. New mathematical modeling frameworks are being developed to handle the huge number of states in which a single signaling protein may be found (10). Proteomic approaches are being developed to quantify site-specific covalent modifications in cell extracts on a large scale (11). While this approach allows large-scale, quantitative analysis of covalent modifications to signaling proteins, it does not gauge subcellular spatial information.

Thus, complementary methods are needed to quantify spatial information on signaling proteins that have undergone site-specific covalent modifications. Classical immunofluorescence (IF) staining provides an excellent starting point. In IF staining, antibodies are used to detect an antigen (e.g., signaling protein) in fixed cells (12). These antibodies may be tagged with fluorophores, including quantum dots that have unique advantages over GFP. Furthermore, antibodies for site-specific covalent modifications are widely available commercially. A limiting factor, however, is that images acquired by IF are primarily analyzed qualitatively. Here, we describe image analysis algorithms that may be used to quantify IF images in an automated manner. As a case study, we apply the algorithms to quantify the level of nuclear extracellular-regulated kinase (ERK) signaling.

Experimental Design

In this work, we developed and tested image analysis algorithms to quantify the spatial localization of phosphorylated signaling proteins. We focused on phosphorylated ERK, a signal that localizes to the nucleus and is required for cell proliferation (13). We performed a dose-dependence assay to gauge how the localized signal responds to different amounts of stimuli. Such dose-response studies provide a well-defined approach to test whether our measurement methodology could discern quantitative changes in signaling.

It is useful to conduct such experiments in systems that have been confirmed to trigger the signal of interest using other experimental assays. Therefore, we chose a stimulus, epidermal growth factor (EGF), that is well known to trigger ERK signaling (14-15). We used MCF-10A cells that respond to EGF by triggering ERK phosphorylation as confirmed by Western blotting (16).

Materials

Cell culture

6 well plate (Corning)

Micro cover glass, 18 mm circle (VWR)

Dulbecco's modified Eagle's medium/Ham's F-12 containing HEPES and L-glutamine (Gibco)

Epidermal growth factor (Peprotech)

Hydrocortisone (Sigma-Aldrich)

Insulin (Sigma-Aldrich)

Choleratoxin (Sigma-Aldrich)

Bovine serum albumin (Sigma-Aldrich)

Trypsin EDTA 0.05% (Gibco)

Penicillin/streptomycin (Gibco)

Buffers/Reagents

Phosphate buffered saline (PBS)

Paraformaldehyde (Sigma-Aldrich)

Tween-20 (Sigma-Aldrich)

Methanol (EMD)

Glycine (Sigma-Aldrich)

Triton X-100 (Sigma-Aldrich)

Goat serum (Gibco)

NP-40 (Sigma-Aldrich)

NaCl (Sigma-Aldrich)

Na_2HPO_4 (Sigma-Aldrich)

NaH_2PO_4 (Sigma-Aldrich)

NaN_3 (Sigma-Aldrich)

PD98059 (Calbiochem)

Na_3VO_4 (Sigma-Aldrich)

NaF (Sigma-Aldrich)

β-glycerophosphate (Sigma-Aldrich)

Immunofluorescence reagents

Primary antibodies:

Phospho-p44/42 MAPK (Thr202/Tyr204)

Polyclonal: #9101 (1:200) and Monoclonal: #4377 (1:50)

(Cell Signaling Technology)

Secondary antibody:

Alexa Flur 488 (1:200) (Molecular Probe)

4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich)

ProLong Gold antifade (Molecular Probe)

Methods

Cell culture and stimulation for phospho-ERK measurements

1. Culture MCF-10A cells in Dulbecco's modified Eagle's medium/Ham's F-12 containing HEPES and L-glutamine supplemented with 5% (v/v) horse serum, 20 ng/mL EGF, 0.5 μg/ml hydrocortisone, 0.1 μg/ml cholera toxin, 10 μg/ml insulin, and 1% penicillin/streptomycin.
2. Plate cells on sterilized glass cover glass placed in the 6-well tissue culture plates at 1 x 10^5 cells per well and grow cells in growth medium for 24 h to allow adhesion.
3. For G_0 synchronization, wash cells twice with PBS and culture them in serum-free medium for 24 h: DMEM/F-12 supplemented with 1% penicillin/streptomycin and 0.1% bovine serum albumin.
4. For EGF stimulation, reconstitute recombinant human EGF in sterile H_2O at 100 μg/ml and dilute it in serum-free medium to designated concentrations.
5. Make sure EGF containing medium is warmed to 37 ^oC. Then stimulate cells for

15 min by adding 2 ml of EGF containing medium to each well. Either cells incubated in the absence of EGF or treated with a pharmacological inhibitor of MEK, PD98059, can be used as a negative control while cells treated with 10 ng/ml can serve as a positive control.

Antibody labeling of ppERK

1. After 15 min of EGF stimulation, place 6-well plates on the ice and wash cells twice with ice-cold PBS.
2. Fix cells in freshly prepared 2% paraformaldehyde (pH 7.4) for 20 min at room temperature in the presence of phosphatase inhibitors at the following concentrations: 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM β-glycerophosphate. Rinse with 0.1 mM solution of Glycine in PBS three times.
3. Permeabilize cells in PBS containing 0.5% NP-40 and the phosphatase inhibitors for 10 min at 4^{o}C with gentle rocking. Rinse with PBS three times.
4. Dehydrate cells in ice-cold pure methanol for 20 min at -20^{o}C. Rinse with PBS three times.
5. Block with IF Buffer: 130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 7.7 mM NaN_3, 0.1% bovine serum albumin, 0.2% Triton X-100, 0.05% Tween-20 and 10% goat serum for 1 h at room temperature.
6. Incubate with anti-phospho-p44/42 MAPK antibody in IF buffer overnight at 4^{o}C. Rinse three times with IF buffer at room temperature on the rocker for 20 min each. Washing step is essential to minimize background staining.

7. Sequentially incubate with Alexa dye-labeled secondary antibodies (Invitrogen-Molecular Probe) in IF buffer for 45 min at room temperature. Rinse three times with IF buffer at room temperature on the rocker for 20 min each. Make sure to protect samples from light.
8. Counterstain nuclei with 0.5 ng/ml DAPI (Sigma-Aldrich) for 15 min at room temperature and rinse with PBS twice with gentle rocking for 5 min each.
9. Mount with ProLong Gold antifade (Invitrogen-Molecular Probe). Dry overnight in a place that can protect samples from light.

Fluorescence microscopy imaging of ppERK and automated image analysis

1. Acquire fluorescence images using filters for DAPI and FITC. Start with a sample that is expected to give the highest FITC signal (e.g., the positive control, 10 ng/ml EGF). Using this positive control, empirically choose an exposure time so that the highest pixel intensity in a given field is close to the saturation level (generally 255). Be sure that the chosen exposure time does not saturate the FITC signal in other fields of the positive control sample. These steps identify an exposure time that maximizes the dynamic range of ppERK signals that may be quantified. The exposure time determined in this way should then be fixed and used to capture images from all other samples.
2. Segment DAPI (nuclei) images using a combination of edge detection and Watershed algorithms. The algorithm to process a single image is written in MATLAB (MathWorks) as described below (steps 2a-e). This algorithm can be iterated to process multiple images in a single execution.

a. Import a DAPI image using *imread* function.

```
DAPI = Imread ( 'DAPI image.tif' )
```

b. *edge* function detects the edge of the objects using gradients in pixel intensity across the objects and returns a binary image where the edge of objects is traced. Different masks are available in *edge* function. 'sobel' and 'canny' methods were successfully used in this study.

```
[edgeDAPI, thresh] = edge ( DAPI, 'sobel')
```

Optionally, *imdilate* and *imerode* functions can be used together to enhance the results of edge detection.

c. Fill in the inside of the traced nuclei using *imfill* function.

```
edgefillDAPI = imfill (edgeDAPI, 'holes')
```

d. Edge detection method often cannot distinguish cells that are spaced too closely. Watershed algorithm can be used along with distance transform to separate merged multiple nuclei. Use of *bwdist* and *watershed* functions will generate an image having lines that would separate touching cells. Optionally, *imhmin* function can be used to prevent oversegmentation which is a known problem of watershed algorithm in some cases. Finally, change the obtained image into the binary image to match the class type.

```
distDAPI = -bwdist (~edgefillDAPI)

  distDAPI2= imhmin (dsitDAPI,1)
```

```
ridgeDAPI= watershed(distDAPI2)

ridgeDAPI2= im2bw(ridgeDAPI)
```

e. Merge two images generated by edge detection algorithm and watershed algorithm to create a single nuclear compartment image.

```
segmentedDAPI = edgefillDAPI & ridgeDAPI2
```

3. Additionally, apply size thresholds to the images to exclude non-cellular objects. The distribution of nucleus size can be approximated as a normal distribution. Thus, use three standard deviations above and below the mean area of nuclei as the upper and lower cut-off values.
4. Using FITC images, calculate the average fluorescence level of the non-cell areas on a per pixel base to account for the background level for each image.
5. Using the segmented images (nuclear mask) and FITC image together, calculate the area of individual nucleus and sum up the FITC values in this area. Finally, the phospho-protein intensity for each cell can be calculated in a following way: multiply the average background level by the area of the nucleus and subtract this value from the total FITC in the nucleus.

$$ppERK = \sum_{nucleus} FITC - \overline{Background} \times AR_{nucleus}$$

Data Acquisition, Anticipated Results, and Interpretations

We quantified the level of phosphorylated ERK (ppERK) in the nucleus of MCF-10A cells that were stimulated with 0.01 or 10 ng/ml EGF or left untreated for 15 minutes. At a qualitative level, the dose-dependent phosphorylation of ERK was evident (Figure 1).

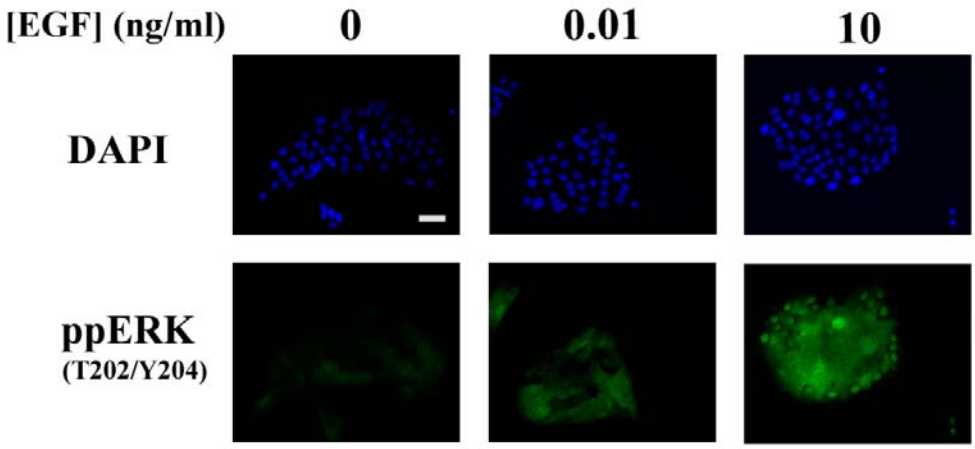

Figure 1. Serum-starved MCF-10A cells were stimulated with 0, 0.01, and 10 ng/ml EGF. Following 15 min of stimulation, cells were immunostained against ppERK (FITC) and nuclei were counterstained with DAPI. The scale bar represents 50 μm

Furthermore, the localization of ppERK to the nucleus was most evident at the highest EGF concentration. The dose-dependent activation of ERK was confirmed using our quantitative image processing algorithms (Figure 2). At the highest EGF concentration, the average amount of nuclear ppERK was approximately five-fold above the response when EGF was absent. Meanwhile, a relatively moderate amount of EGF (0.01 ng/ml) induced only a 3-fold increase in nuclear ppERK.

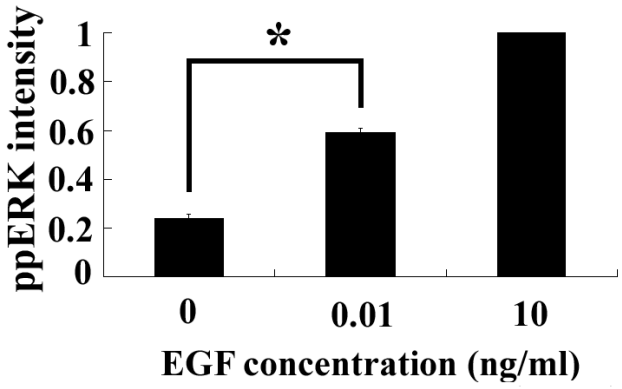

Figure 2. Average nuclear ppERK intensities in samples treated with 0, 0.01, and 10 ng/ml EGF. The *error bars* indicate S.E. (n=3) with duplicates performed in each experiment. The *asterisk* denotes $p < 0.01$ (Student's *t* test)

Since these measurements were conducted at the single-cell level, one can analyze the variation in cell responses across the population. We generated a histogram representing the distribution of nuclear ppERK levels across the population for the three different EGF concentrations (Figure 3). In the absence of EGF, most cells fall into a narrow range of low nuclear ppERK intensity. As the EGF concentration was increased, this distribution shifted gradually to the right. These results indicate that the level of

nuclear ppERK is a graded response to EGF stimulation at the single-cell level.

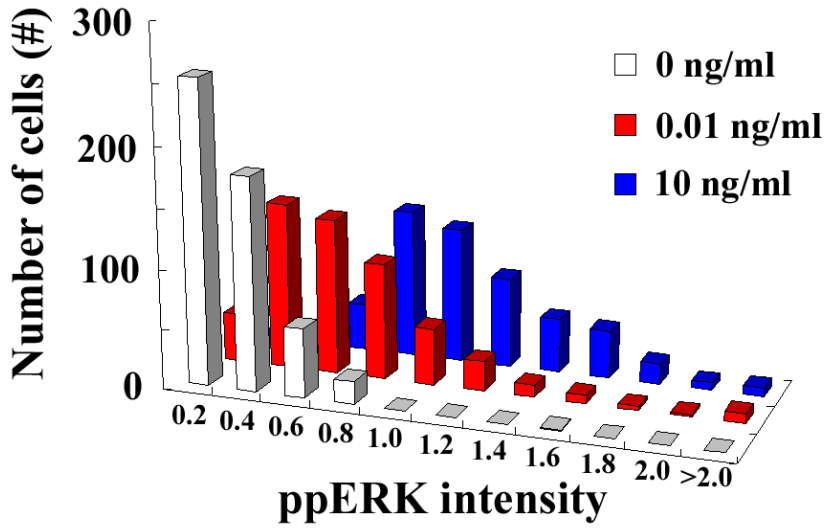

Figure 3. Histogram representation of the distribution of nuclear ppERK levels in cell populations treated with 0, 0.01, and 10 ng/ml EGF.

Statistical Guidelines

Total of three independent trials (n=3) were conducted to gather statistically meaningful data. In each trial, duplicates were prepared for each condition to minimize errors associated with sample preparation. For each sample, five images were collected at the multiple fields. All together, we analyzed at least 150 cells for each condition.

In each trial, the average amount of nuclear ppERK for each condition was expressed relative to the level in 10 ng/ml EGF sample. Thus, a statistical test was not performed between the 10 ng/ml EGF sample and other samples. One tailed-Student's *t* test was performed between 0 and 0.01 ng/ml EGF sample and indicated that these values

were different with a p-value less than 0.01. Error bars represent standard error with n = 3.

Discussion and Commentary

Intracellular signaling pathways control cell behaviors and multicellular morphodynamics. A quantitative understanding of these pathways will provide design principles for tuning these signals in order to engineer cell behaviors and tissue morphology. The transmission of information in these pathways involves both site-specific covalent modifications to signaling proteins and spatial localization of these signals. Here, we describe algorithms for quantifying signal localization from immunofluorescence (IF) staining for phosphorylated ERK. Our data reveal that in epithelial cells, ERK exhibits a graded response to EGF not only at the population level, but also at the level of individual nuclei. These results are consistent with the other studies that reported graded ERK responses to various stimuli in other mammalian cell systems (17-18). The algorithms presented here should facilitate quantitative, high throughput analysis of images acquired by IF staining.

Troubleshooting Table

Problem	**Explanation**	**Potential Solutions**
Background is too high	- Nonspecific binding of primary or secondary antibody	- Make sure to follow the required blocking and washing steps thoroughly.

	- Basal ERK activity mediated by autocrine factor.	- Perform a negative control using only the secondary antibody and skipping the primary antibody incubation to assess the level of nonspecific binding. - Prepare a sample treated with PD98059 to quench ERK activity all together.
The number of segmented nuclei is significantly less than the actual number of nuclei.	- Failure to detect the edge of some of the nuclei	- Increase the exposure time until DAPI signals at the location of nuclei become saturated. It will ensure the contrast between nuclei and the background. - Alternatively, *imadjust* or *contrast* algorithms can be used in MATLAB to enhance the contrast of a DAPI image before performing nuclear segmentation.
The number of nuclei is	- Many non-cellular objects	- Rinse and wipe the slides

significantly overcounted.	were considered as nuclei. - Oversegmentation from watershed algorithm.	with alcohol to get rid of dried salts and stain. - Avoid air bubbles when mounting the sample with antifade. - Adjust the upper and lower limits of area threshold of nuclei appropriately to exclude non-cellular objects with qualitative verification. - Use *imhmin* function to reduce oversegmentation.

Application Notes

The method described in this report would be particularly useful in quantifying the spatiotemporal signaling response at a single-cell level. The algorithm should allow automated and high throughput quantification of subcellular compartmentation of signaling events in response to multiple combinations and doses of environmental stimuli. It should also prove useful for quantitative studies of cell-to-cell variation in signaling. Such measurements would provide valuable quantitative data for systems-level analysis of signal transduction networks, the regulatory architecture that governs cellular decision-making.

Summary Points

- Before beginning image acquisition, choose an exposure time that maximizes the dynamic range of signals that may be quantified. Chosen exposure time should be fixed and used to capture images from all the samples.
- Qualitatively verify that the edge detection and watershed algorithms properly segment individual nuclei.
- Size thresholds are often necessary to exclude non-cellular objects.
- Account for background fluorescence level to measure exclusively fluorescence signals from signaling proteins.
- Choose a proper sample size (e.g., the number of cells analyzed in each trial), depending on the degree of cell-to-cell variance of the target proteins.
- Add phosphatase inhibitors at fixation and permeabilization steps if target signal molecules are phospho-proteins.
- Rigorous washing after incubation with antibodies is essential to minimize background staining.

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