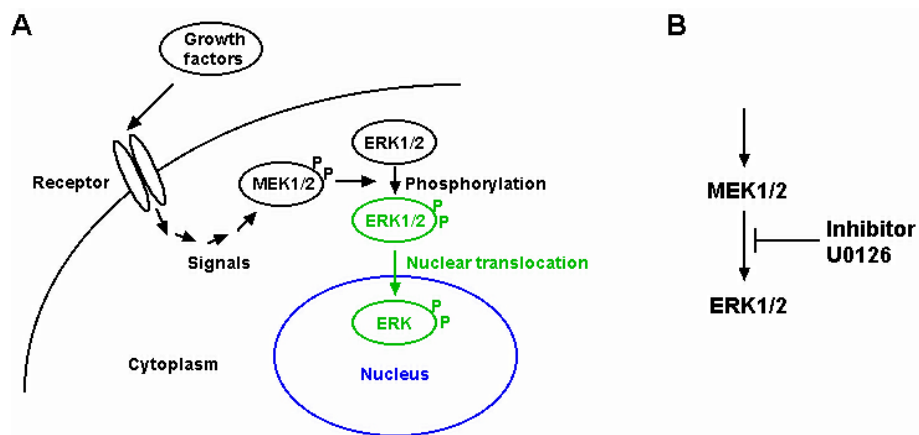


## Introduction

The intracellular signal transduction mechanism plays a significant role in various cellular events such as differentiation, growth and stress response. Western blotting is one of the techniques most often used to assay these cellular reactions. Although western blotting is superior for specifically tracking the kinetics of desired protein molecules, it has disadvantages in throughput and precise quantification. To address this problem, we tested the feasibility of using an image-based analytic approach for assaying signal transduction molecules. We selected ERK1 and ERK2 molecules as a model experiment. ERK1 and 2 are MAP kinase family members and are key players in many signal transduction pathways. Upon activation, these two molecules are dual-phosphorylated by upstream kinases (MEK1 and 2) and translocate from the cytoplasm to the cell nucleus (Figure 1A).<sup>1</sup>



**Figure 1: Schematic drawing of an ERK1/2 mediated signal transduction pathway (A) and a target point of U0126 (B)**

## Experimental Objective

Using the iCyte™ Automated Imaging Cytometer, we measured the amount of phosphorylated and nuclear-translocated ERK1/2. To create various activity levels of ERK1/2 in CHO cells, we treated the cells with fibroblast growth factor-1 (FGF-1) and U0126. FGF-1 stimulates activation of the ERK-mediated signal transduction pathway by binding to its extracellular receptors. U0126 is known to inhibit activation of ERK1/2 by MEK1/2 (Figure 1B) (<sup>2, 3</sup>). We also compared iCyte results with data from a western blotting analysis to confirm the agreement of the two experimental protocols.

## Materials

### Cell Line

CHO-WBLT cells (a derivative of CHO-WBL) maintained in McCoy's 5A medium containing 10% FBS

**Reagents**

Reagent	Source
1. McCoy's 5A Medium Modified	GibcoBRL, cat. no. 16600-082
2. Trypsin	GibcoBRL, cat. no. 15050-065
3. Fetal bovine serum (FBS)	Sigma, cat. no. F2442
4. Fibroblast Growth Factor, Acidic, Human, Recombinant (FGF-1)	Promega, cat. no. G5061
5. MEK Inhibitor U0126	Promega, cat. no. V1121
6. Anti-ACTIVE <sup>®</sup> -MAPK pAb (rabbit made)	Promega, cat. no. V8031
7. Anti-rabbit IgG FITC conjugate (goat made)	Sigma, cat. no. F0382
8. Albumin, Bovine, Fraction V (BSA)	Sigma, cat. no. A-6003
9. Hoechst 33258	Molecular Probes, cat. no. H-1398
10. 1,4-diazabicyclo[2.2.2]octane (DABCO)	Wako, cat. no. 049-25712
11. Dimethylsulfoxide (DMSO)	Wako, cat. no. 349-01025
12. Methanol	Wako, cat. no. 131-01826
13. RNase A	Wako, cat. no. 47003A

**Sample Carrier**

96-well plastic-bottom microplate, Whatman cat. no. 7716-2380

**Cell Media**

McCoy's 5A Medium supplemented with 10% FBS. Use serum-free medium for serum starvation.

**Blocking Solution**

PBS containing 1% BSA

**Fixative Solution**

Six percent (6%) formaldehyde solution in PBS. To prepare this solution, warm 10 ml of PBS to approximately 60°C in a microwave oven. Add 0.6 g of paraformaldehyde to the PBS, mixing thoroughly with a magnetic stirrer, then allow to cool to room temperature. *This solution should be prepared under a ventilator.*

## Instrumentation

iCyte™ Automated Imaging Cytometer, CompuCyte Corporation, installed with iCyte™ software version 2.1.2 and iBrowser™ data integration software version 2.1.2.

## Additional Analysis Tools

Microsoft® Excel  
GraphPad Prism® version 4 (GraphPad Software)

## Experimental Method

### Plate Preparation

1. Trypsinize actively growing CHO cells.
2. Centrifuge the cells and re-suspend in fresh cell media at a density of  $1 \times 10^5$  cells/ml.
3. Add 100  $\mu$ l of the cell suspension to each well of a 96-well microplate.
4. Incubate plates at 37°C and 5% CO<sub>2</sub> for 6 hours to allow cells to attach to the well bottom.
5. Aspirate cell media and wash the cells with 200  $\mu$ l of serum-free media. Repeat twice more.
6. Aspirate washing media and add 200  $\mu$ l of fresh serum-free media.
7. Incubate plates at 37°C and 5% CO<sub>2</sub> overnight for serum starvation.

### Cell Treatment

#### ***For FGF-1 stimulatory activity measurement (See Figure 2):***

8. Dilute FGF-1 solution to 100 ng/ml in dilution buffer (PBS plus 1% BSA).
9. Prepare 5-fold serial dilution of FGF-1 solution (100 ng/ml – 1.28 pg/ml).
10. Add 10  $\mu$ l of the FGF-1 solutions to the plate wells in triplicate, starting with the highest concentration in column 11 (final 5 ng/ml) and reducing to the lowest concentration in column 4 (final 64 fg/ml).
11. Add 10  $\mu$ l of dilution buffer to column 3 to make negative control wells.
12. Incubate the plate at 37°C for 10 minutes.
13. Add 100  $\mu$ l of 6% formaldehyde fixative to the wells (to give a final concentration of 2%) to stop the reaction.

(Proceed to step 14 below.)

#### ***For U0126 inhibitory activity measurement (See Figure 3):***

- 8'. Prepare 40 mM U0126 solution in DMSO.
- 9'. Prepare 5-fold serial dilution of U0126 in DMSO (4 mM – 51 nM).
- 10'. Add 5  $\mu$ l of U0126 solutions to the plate wells in triplicate to give final concentrations from 100  $\mu$ M (column 11) to 1.3 nM (column 4).
- 11'. Add 5  $\mu$ l of DMSO in column 3 in triplicate to prepare control wells.

- 12'. Incubate at 37°C for 30 minutes to allow U0126 molecules to penetrate into the cells.
  - 13'. Prepare 200 pg/ml FGF-1 solution in PBS containing 1% BSA.
  - 14'. Add 10 µl of FGF-1 solution to the wells to give final concentration of 10 pg/ml.
  - 15'. Incubate the plate at 37°C for 10 minutes.
  - 16'. Add 100 µl of 6% formaldehyde fixative to all of the pre-treated wells (to give final concentration of 2%) to stop reaction, giving a final concentration of 2%.
- (Proceed to step 14 below.)

**Technical Hint**

*The FGF-1 concentration for the U0126 inhibitor assay (plate 2) is determined based on an EC<sub>50</sub> value estimated from the plate 1 experiment.*

**Cell Staining****Antibody Preparation**

14. Continue formaldehyde fixation for 15 minutes at room temperature.
15. Wash wells one time with 200 µl of PBS.
16. Add 200 µl of methanol to the wells and incubate at room temperature for 10 minutes.
17. Wash wells one time with 200 µl of PBS.
18. Add 200 µl blocking solution and incubate at room temperature for 15 minutes. Aspirate the blocking solution.
19. Prepare anti-ACTIVE<sup>®</sup>-MAPK antibody at 1:500 in blocking solution.
20. Add 60 µl of diluted antibody to each well.
21. Incubate the plate for at least 2 hours at room temperature (or overnight at 4°C).

**Antibody Staining**

22. Wash wells two times with 200 µl PBS.
23. Prepare anti-rabbit IgG FITC conjugate at 1:100 in blocking solution.
24. Add 60 µl of diluted antibody to each well.
25. Incubate the plate at room temperature for one hour.
26. Wash wells two times with 200 µl PBS.

**Counterstaining**

27. Counterstain cells by adding 200 µl of cocktail containing 5 ng/ml Hoechst 33258, 100 ng/ml RNase and 1% DABCO in PBS. Incubate at room temperature for 10 minutes.

**Technical Hint**

*DABCO is supplemented as an antifade reagent against FITC photo-bleaching.*

**Plate Set-up**

Two types of plate setups are indicated in Figures 2 and 3.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			Vehicle	64 fg/ml	320 fg/ml	1.6 pg/ml	8 pg/ml	40 pg/ml	200 pg/ml	1 ng/ml	5 ng/ml	
C			Vehicle	64 fg/ml	320 fg/ml	1.6 pg/ml	8 pg/ml	40 pg/ml	200 pg/ml	1 ng/ml	5 ng/ml	
D			Vehicle	64 fg/ml	320 fg/ml	1.6 pg/ml	8 pg/ml	40 pg/ml	200 pg/ml	1 ng/ml	5 ng/ml	
E												
F												
G												
H												

**Figure 2: 96-well microplate setup for the FGF-1 stimulatory activity assay (Plate 1). Final FGF-1 concentrations are indicated on the panel.**

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			Vehicle	1.3 nM	6.4 nM	32 nM	160 nM	800 nM	4 μM	20 μM	100 μM	
C			Vehicle	1.3 nM	6.4 nM	32 nM	160 nM	800 nM	4 μM	20 μM	100 μM	
D			Vehicle	1.3 nM	6.4 nM	32 nM	160 nM	800 nM	4 μM	20 μM	100 μM	
E												
F												
G												
H												

**Figure 3: 96-well microplate setup for U0126 inhibitory effect assay (Plate 2). Final U0126 concentrations are indicated on the panel. After incubation in the presence of U0126, add FGF-1 (10 pg/ml) to all treated wells.**

**Western Blotting Analysis**

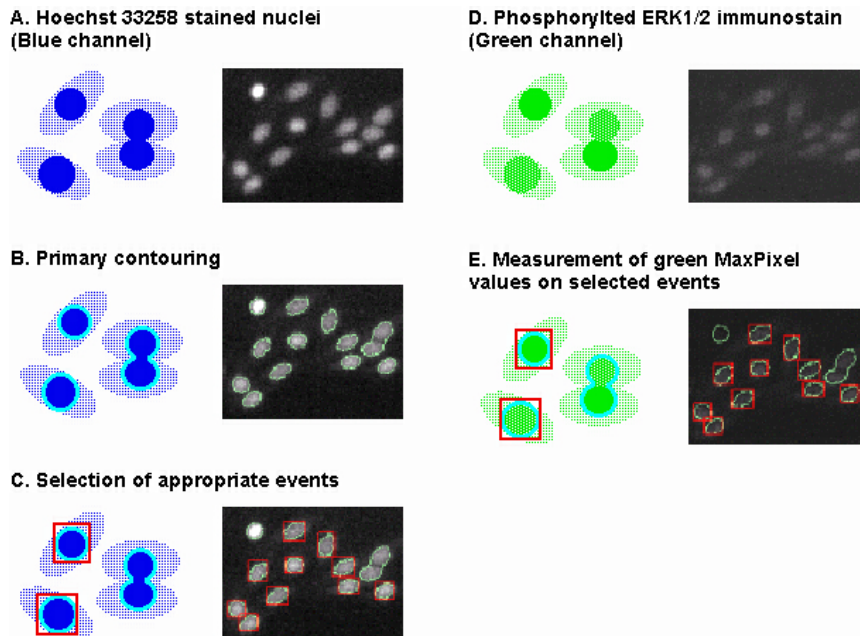
1. Seed actively growing CHO cells in a 24-well microplate at a density of  $1 \times 10^5$  cells/well.
2. Serum-starve the cells overnight with serum-free culture media.

3. Challenge cells with serial dilutions of FGF-1 to final concentrations from 10 ng/ml to 10 fg/ml and incubate at 37°C for 10 minutes.
4. Aspirate supernatant and quickly lyse the cells by adding lysis buffer (10 mM sodium phosphate, pH 6.8, 1% SDS and 7M urea). Transfer the lysate to a small centrifuge tube and sonicate to shear chromosomal DNA.
5. Separate the cell lysate by 10% SDS-polyacrylamide gel electrophoresis, then semi-dry blot onto a PVDF membrane.
6. Probe dual-phosphorylated ERK1/2 proteins with Anti-ACTIVE®-MAPK antibody.
7. Detect bound primary antibody by anti-rabbit IgG HRP conjugate (Promega) and ECL+Plus™ (Amersham Biosciences).
8. To detect total number of ERK1/2 molecules on the blot, re-probe the membrane with rabbit anti-ERK1 (K-23) antibody (SantaCruz) and repeat step 7.

### Sample Analysis

#### Introduction

Nuclear areas are targeted by contouring Hoechst 33258-stained nuclei on the Blue2 channel (primary contour). (See Figure 4 A, B, and C.) The active form of ERK1/2 molecules are labeled with FITC and measured in the Green channel. Green MaxPixel values on selected primary contours are measured and their mean values are determined (Figure 4 D and E).



**Figure 4: Schematic illustration of image analysis algorithm for quantitative measurement of ERK1/2 activity**

## Initial Set-up

1. Load the FGF-1 treated microplate (Plate 1).
2. The iCyte instrument settings are as follows:

Primary Laser	Argon 5 mW
Channel	Green
Secondary Laser	Violet
Channel	Blue 2
Objective	40x
X-step Size	0.5 microns

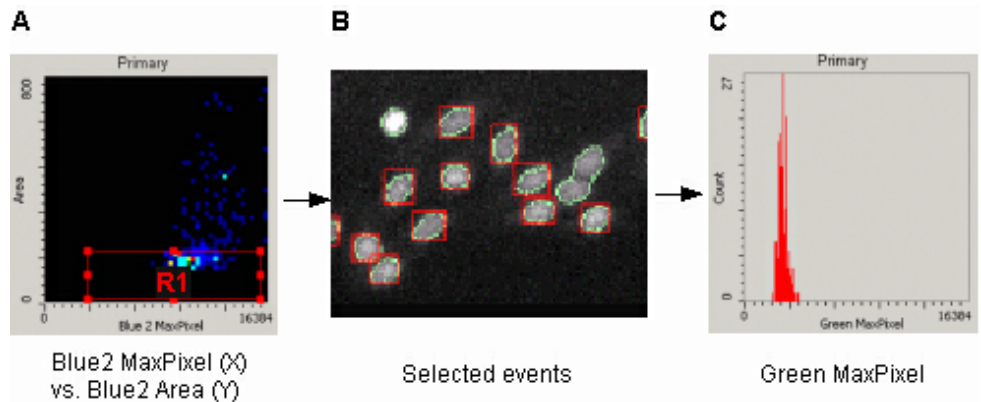
## Scan Settings Set-up (Test Scans)

3. Select a negative control well without FGF-1 treatment.
4. Select a scan area of 6 (wide) x 6 (high).
5. Set the primary segmentation parameters as indicated below:

Primary Segmentation	Blue2
Threshold	3000
Minimum area	30 $\mu\text{m}^2$
Add pixels	0
Background	Manual

## Analysis Scan Set-up

6. Define the following windows:
  - A scattergram for Blue2 MaxPixel (X-axis) vs. Area (Y-axis). Create a rectangular region "R1" on the scattergram (Figure 5A).
  - A histogram for green MaxPixel of events included in R1 (Figure 5C).
7. Start a test scan. Confirm that the threshold value for primary segmentation is properly set for precise contouring of cell nuclei.
8. Adjust X- and Y-axis ranges of the region R1 on the scattergram to remove M-phase and apoptotic cells (Blue2 MaxPixel values) and aggregated cells (high Blue2 Area values). (See Figures 5A and 5B.)



**Figure 5: Selection of primary contoured nuclei (A and B) and a histogram of their green MaxPixel values (C)**

### Data Acquisition

9. Set the Data Options to save raw data as a JPEG file.
10. Set Well Features as indicated in Figure 6. The mean MaxPixel values of the Green channel are used for further statistical analysis.
11. Select Scan and Save.

Component	Feature	Channel	Stat	Region	Min	Max	Valid
Primary			Count	R1	0	1000	True
Primary	MaxPixel	Green	Mean	R1	0	16000	True

**Figure 6: Well Features setting to obtain analysis data for further statistical analysis**

### Data Analysis

12. After the scan is completed, open the iBrowser program.
13. Transfer numerical data indicated in iBrowser to an Excel spreadsheet and save the file.
14. Transfer the numerical data to GraphPad Prism<sup>®</sup> software for statistical analysis.

## Results

### iCyte Analysis

By incubating the cells in the absence of FBS, the cellular signal transduction cascade goes into a quiescent state and therefore the majority of the ERK1/2 molecules are not phosphorylated. In the negative control wells in Plate 1, the cells show poor staining with the antibody that recognizes the phosphorylated structure of these proteins (Figure 7A).



**Table 1. Average Green MaxPixel values of primary contoured events on immunostained CHO cells. A: FGF-1 stimulatory effect (Plate 1), B: U0126 inhibitory effect against FGF-1 (Plate 2).**

<b>A</b>			
FGF-1 (g/ml)	Average MaxPixel		
	Row B	Row C	Row D
0	2964	2972	3015
6.40E-14	2965	2951	3023
3.20E-13	3062	3013	3098
1.60E-12	3194	3186	3204
8.00E-12	3559	3797	3771
4.00E-11	4310	4209	4300
2.00E-10	4133	4154	4195
1.00E-09	3810	3875	3744
5.00E-09	3650	3568	3504

<b>B</b>			
U0126 (M)	Average MaxPixel		
	Row B	Row C	Row D
0	3429	3442	3353
1.3E-09	3432	3374	3286
6.4E-09	3445	3324	3198
3.2E-08	3308	3386	3210
1.6E-07	3330	3283	3259
8.0E-07	2922	2751	2799
4.0E-06	2523	2498	2466
2.0E-05	2534	2501	2463
1.0E-04	2545	2502	2452

Next, transfer the rearranged data to GraphPad Prism<sup>®</sup>. Draw dose-response curves by fitting a non-linear regression (sigmoid) curve. Determine the EC<sub>50</sub> value of the FGF-1 stimulatory (Plate 1) effect (Figure 9A). Note that a high dose of FGF (>10<sup>-10</sup> g/ml) causes a negative effect. Similarly, determine the IC<sub>50</sub> value of the U0126 inhibitory effect (Figure 9B).

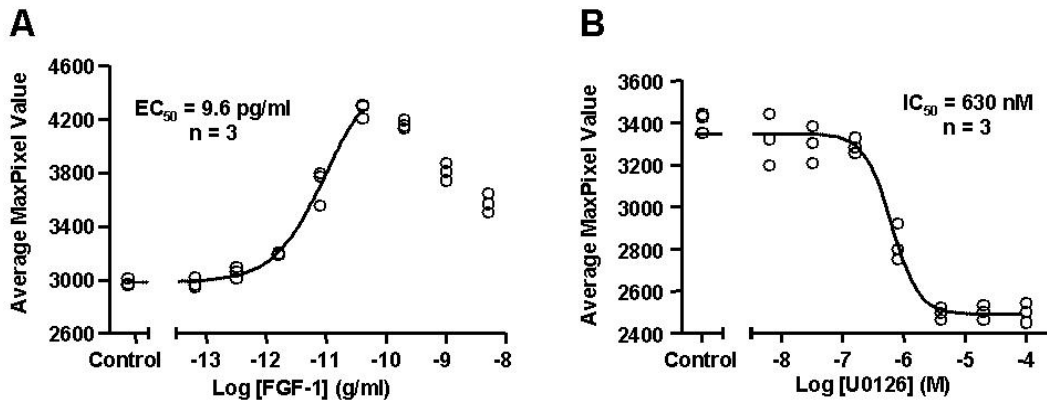


Figure 9: Dose-response curves of FGF-1 stimulatory effect (A) and U0126 inhibitory effect (B)

### Comparison with Western Blotting Data

An analysis of the western blotting using Anti-ACTIVE<sup>®</sup>-MAPK antibody shows a dose-dependent stimulatory response of FGF-1 (Figure 10). The dose-response patterns determined by western blotting and iCyte analysis (Figure 9A) are very similar.

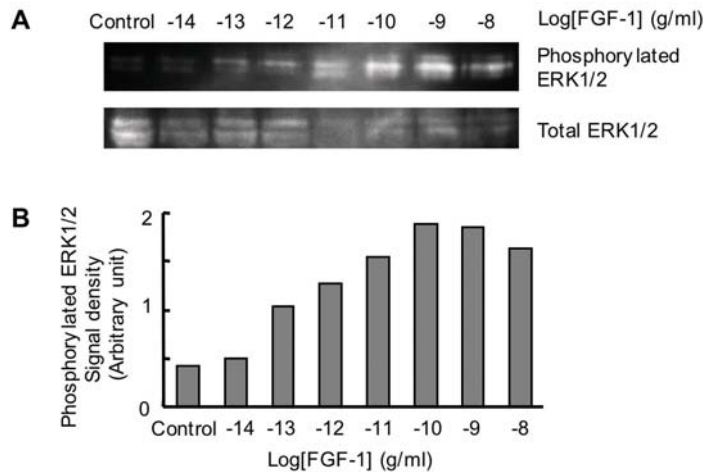


Figure 10: FGF-1 dose-dependent ERK1/2 reaction measured by western blotting analysis

### Summary

In this experiment, we have tested the capability of an image analysis approach for assaying intracellular signal transduction reactions focusing on ERK1/2 molecules.

Upon stimulation of the signal-transduction pathway, ERK1/2 molecules are dual-phosphorylated and translocate to cell nuclei. This phenomenon was specifically and quantitatively measured by

immunofluorescence staining, using a phosphorylated peptide sequence-specific antibody and measuring the intensity of its fluorescence within the nuclear contours.

By using MaxPixel values to represent immunostaining intensities, we obtained a dose-response curve of an FGF-1 stimulatory effect. The result measured by iCyte is consistent with the western blotting analysis data probed with the same primary antibody. We also measured the inhibition activity of a U0126 MEK inhibitor against the FGF-1 stimulatory effect. A sigmoid dose-response curve was obtained and this was suitable for estimation of an IC<sub>50</sub> value.

The results in this Protocol indicate that it is possible to measure ERK1/2-mediated intracellular signal-transduction pathways on the iCyte. This assay protocol has greater quantitative accuracy and higher throughput than the conventional western blotting method.

### Features Summary

Feature	Use during this study
Multi-channel analysis	Acquisition of overlapping green and blue fluorescence images
Automated analysis	Data collection from multiple wells of 96-well microplates
MaxPixel feature	Measurement of condensation of phosphorylated ERK1/2 molecules to cell nuclei
Well Features	Display of average MaxPixel values for further statistical analysis

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### References

- <sup>1</sup> Pearson G et al, Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* (2001) 22: 153-83.
- <sup>2</sup> Duncia JV et al, MEK inhibitors: the chemistry and biological activity of U0126, its analogs, and cyclization products. *Bioorg Med Chem Lett* 8: 2839-44.
- <sup>3</sup> Favata MF et al, Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* (1998) 273: 18623-32.