


A Quantitative Cell-Based High-Content Screening Assay for the Epidermal Growth Factor Receptor-Specific Activation of Mitogen-Activated Protein Kinase



Richik N. Ghosh, Linnette Grove, and Oleg Lapets

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Current Literature Presentation
Jelena
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MAPK – basic facts

- **Protein kinases** are enzymes that **covalently attach phosphate** to the side chain of either **serine, threonine, or tyrosine** of specific proteins inside cells.
- **Phosphorylation of proteins** can control:
 - 1) enzymatic activity
 - 2) interaction with other proteins and molecules
 - 3) location in the cell
 - 4) propensity for degradation by proteases
- **MAPKs phosphorylate** specific **serines** and **threonines** of target protein substrates and regulate:
 - 1) gene expression,
 - 2) mitosis,
 - 3) movement,
 - 4) metabolism,
 - 5) programmed cell death.

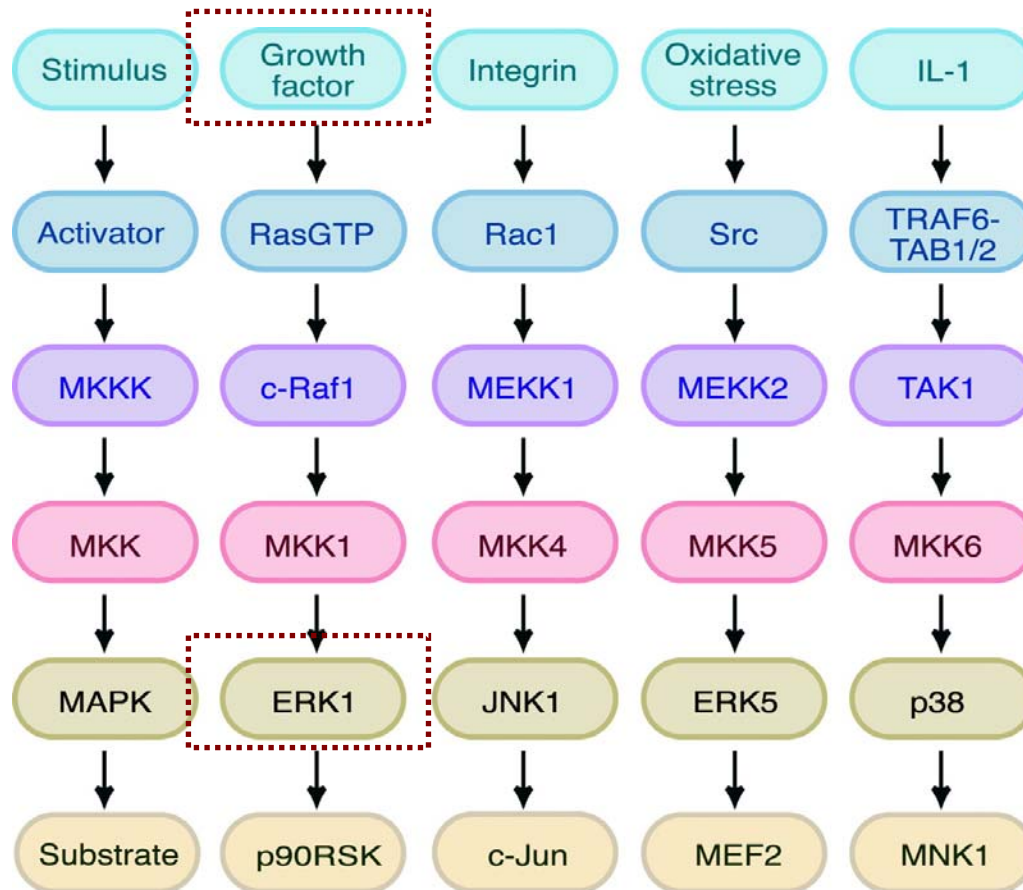
MAPK – basic facts – cont.

- MAPK-catalyzed phosphorylation of substrate proteins functions as a **switch** to turn **on or off** the **activity of the substrate protein**.
- Substrates include:
 - 1) other protein kinases,
 - 2) phospholipases,
 - 3) transcription factors,
 - 4) cytoskeletal proteins.
- ❖ Protein phosphatases remove the phosphates that were transferred to the protein substrate by the MAPK. In this manner, the action of MAPKs and protein phosphatases reciprocally and rapidly alter the behavior of cells as they respond to changes in their environment.

MAPKs subfamilies

- ❖ *Extracellular signal-regulated kinases: ERK1 and ERK2*
 - ❑ involved in the regulation of meiosis, mitosis, and postmitotic functions in differentiated cells
- ❖ *c-Jun NH2-terminal kinases: JNK 1, JNK 2, and JNK 3*
 - ❑ isolated and characterized as stress-activated protein kinases on the basis of their activation in response to inhibition of protein synthesis
- ❖ *p38 enzymes: p38 α , p38 β , p38 γ , and p38 δ*
 - ❑ regulate the expression of many cytokines
 - ❑ activated in immune cells by inflammatory cytokines and has an important role in activation of the immune response
 - ❑ activated by many other stimuli, including hormones, ligands for G protein-coupled receptors, and stresses such as osmotic shock and heat shock.

MAPK phosphorelay systems



 **This is far from linear!**

Extracellular signal-regulated kinase: ERK1 and ERK2

- ❖ ERK1 and ERK2 are proteins of 43 and 41 kDa that are nearly 85% identical overall, with much greater identity in the core regions involved in binding substrates
- ❖ The **two phosphoacceptor sites, tyrosine and threonine**, which are phosphorylated to activate the kinases, are separated by a glutamate residue in both ERK1 and ERK2 to give the motif TEY in the activation loop

- Widely expressed
- Involved in the regulation of meiosis, mitosis, and postmitotic functions in differentiated cells.
- Many different stimuli, including **growth factors**, cytokines, virus infection, ligands for heterotrimeric guanine nucleotide– binding protein (G protein)–coupled receptors, transforming agents, and carcinogens, activate the ERK1 and ERK2 pathways.
- ERKs 1 and 2 are both components of a three-kinase phosphorelay module that includes the MKKK c-Raf1, B-Raf, or A-Raf, which can be activated by the proto-oncogene Ras.

Growth factors activate ERKs

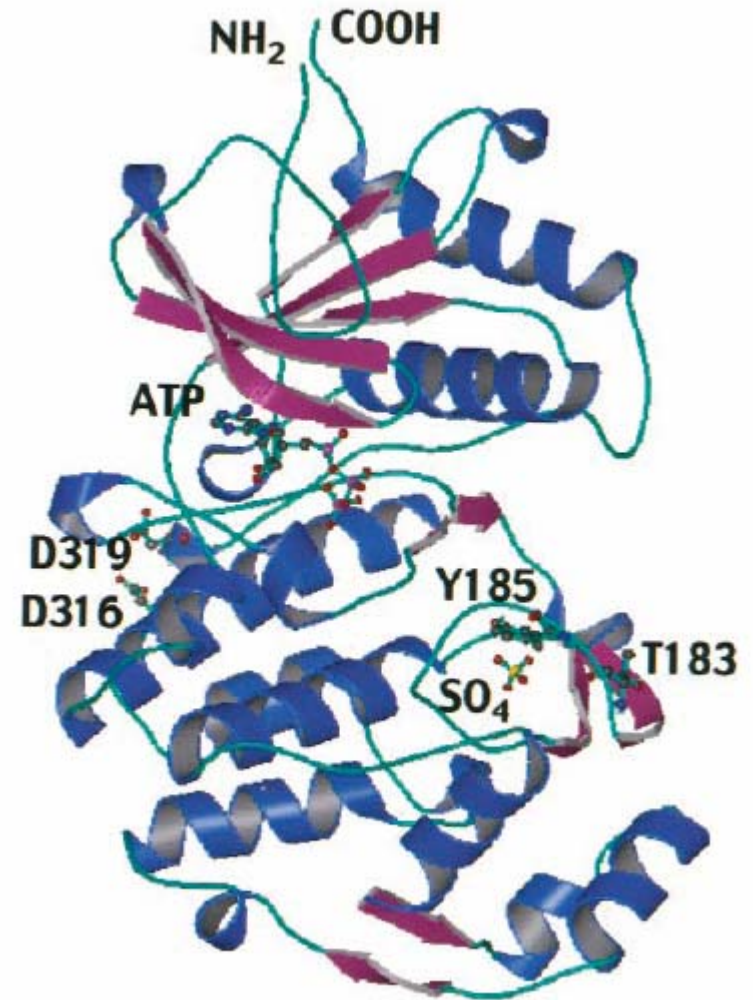
- In response to epidermal growth factor (EGF), the ERK2 translocates into the nucleus.
- When ERK2 is activated in the cytoplasm, it moves to the nucleus after it is phosphorylated by a mechanism, which has been reported to include at least two pathways:
 1. passive diffusion of a monomer and
 2. active transport of a dimer

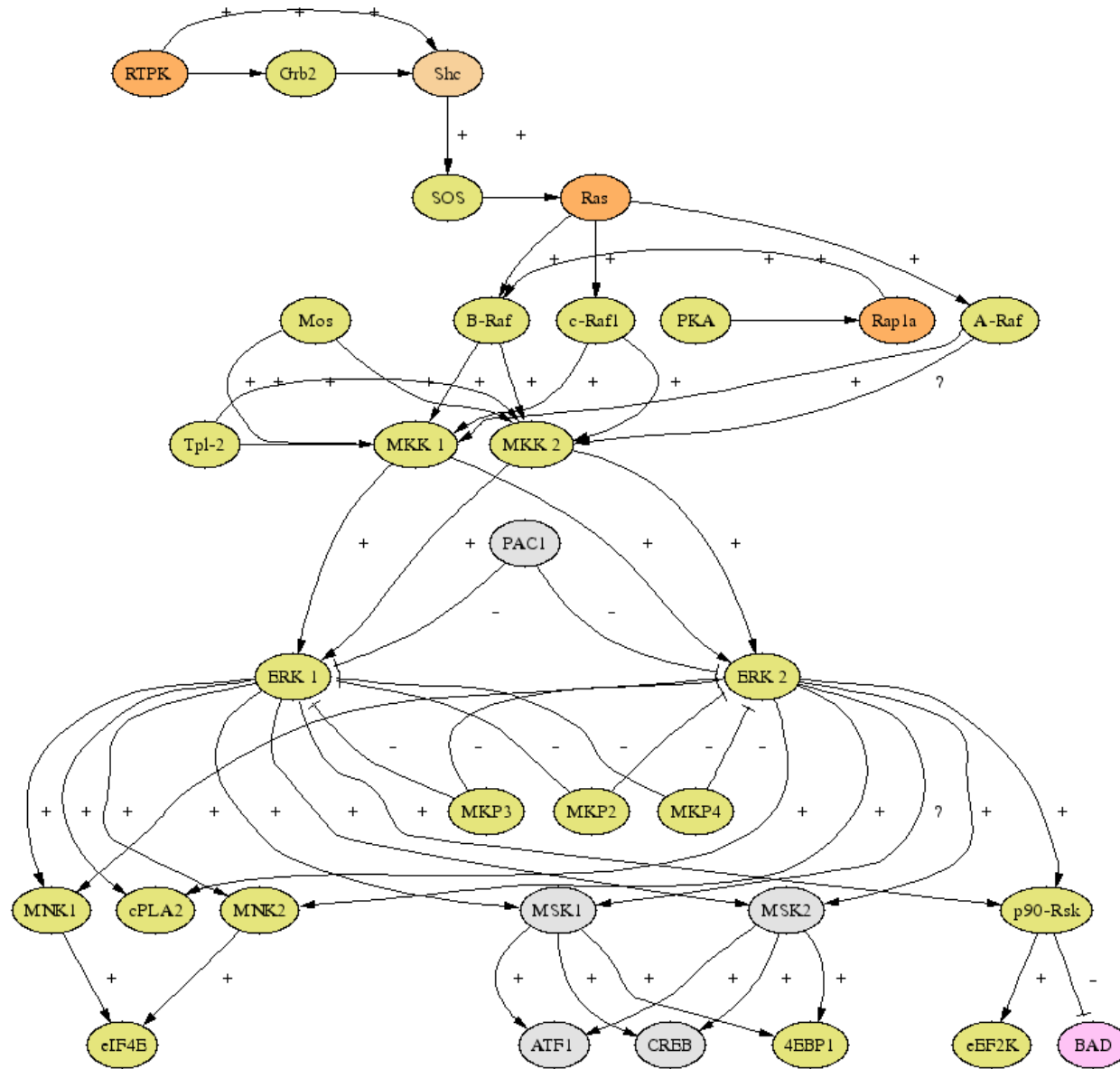
The EMBO 1999, 18, 5347-5358

ERK2 tagged with GFP enters nucleus in a saturable, time and temperature dependent manner and involves a direct interaction with nuclear pore complex proteins.

Phosphorylation of ERK-2

- ATP binds in the interior of the active site at the domain interface and protein substrates are bound on the surface.
- MAP kinase activity is controlled by phosphorylation of two residues, a **tyrosine (185)** and a **threonine (183)**, that are in a surface loop known as the activation loop or phosphorylation lip.





High content analysis

- High-content screening (HCS) is the measurement of complex cellular responses in a population of whole cells in parallel.
 - **Traditional methods:** homogenous *in vitro* assays and expressed proteins in cell based assays
 - investigate protein activity in a well-controlled manner,
 - overall relevance to protein function in the whole-cell environment may be questioned.
 - **HCS** methodology: to assess complex, multi-component targets that cannot be addressed in biochemical assays.
 - The principal advance in cell-based screening
 - ***Able to directly visualize individual cells within multi-well plates***, as an alternative to bulk fluorescence, microscopic and flow cytometry methods.
- ❖ The term '**high content**' is used to refer to processes that are defined spatially and temporally in the context of the structural and functional integrity of each individual cell with an array of cells.

Technological solutions

❖ **Fluorescence microscopy:**

- high resolution,
- high information content,
- huge data files (tera-bites)
- best for validating primary screening hits.

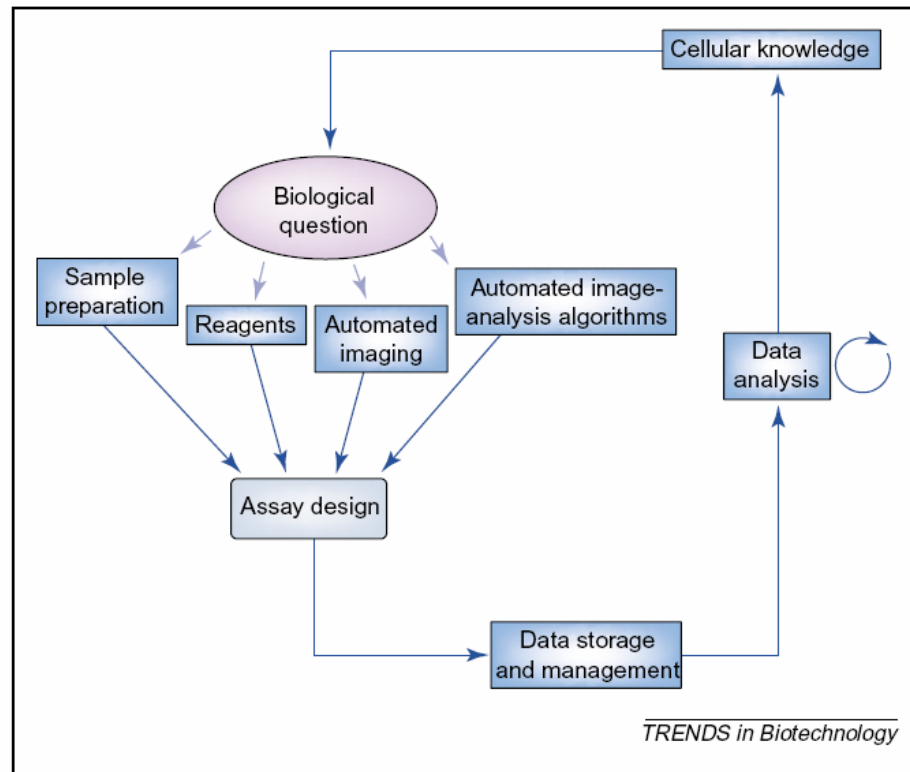
❖ **Laser-scanning cytometry:**

- can be focus-independent
- process data directly to rapidly detect and quantify all fluorescent objects.
- exceptionally fast read times (down to 3 minutes per plate)
- used to identify objects

❖ Analysis of **multicolour fluorescence** in **fixed and/or live cells** by detecting cellular events using **fluorescently tagged markers**

- ❖ Multiple cellular readouts within a single experiment.
- ❖ Fluorescent biomolecules employed as intracellular protein markers are a major advance for HCS.

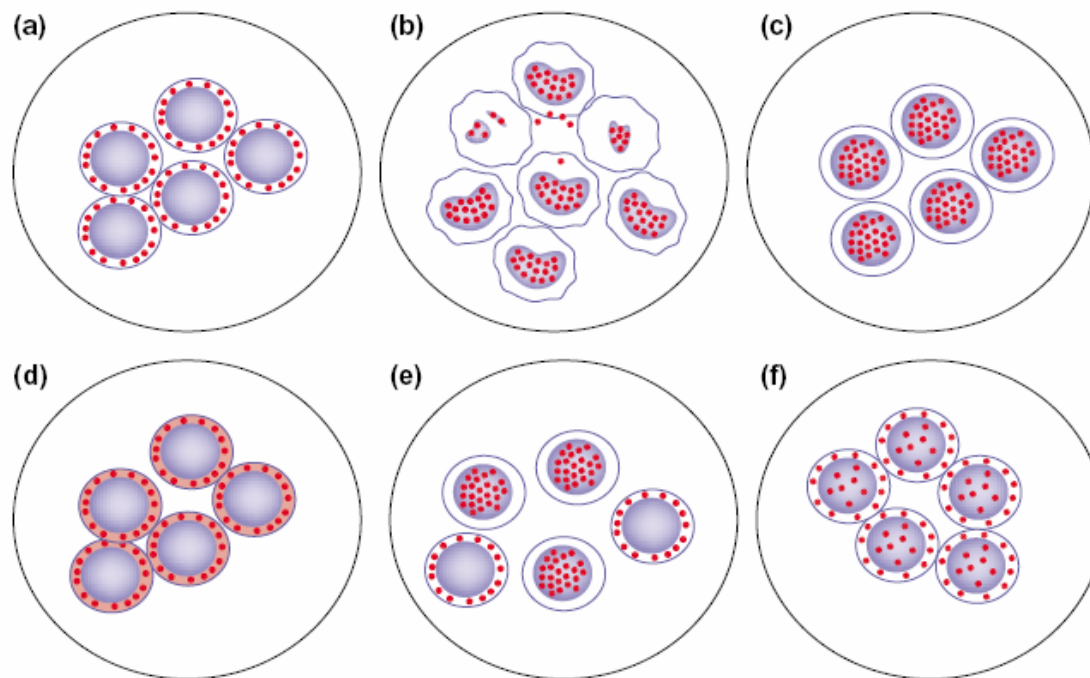
The flow from biological question to data acquisition and analysis and generation of cellular knowledge



- ❑ The cycle must be iterated to build on available cellular knowledge. Fully integrated HCS tools are required to automate the processes described for performance on a large scale. Data analysis is represented as an iterative process to reflect the extraction of different kinds of cellular information from the same set of image data.

HCS is an integration!

- 1) **Fluorescent cell-based assays** where cells are labeled with fluorescent probes that reflect physiological conditions of the cell,
- 2) **Automated, high-resolution fluorescence microscopy** of the fluorescent cells
- 3) **Image processing algorithms - BioApplications**, that automatically analyze the acquired images and report quantitative features reflecting the biological state of both individual cells and all the cells in the population, and
- 4) **Advanced informatics software** that archives and organizes the acquired images and quantitative analysis results, and enables the scientist to easily review these to assist his or her decision making.

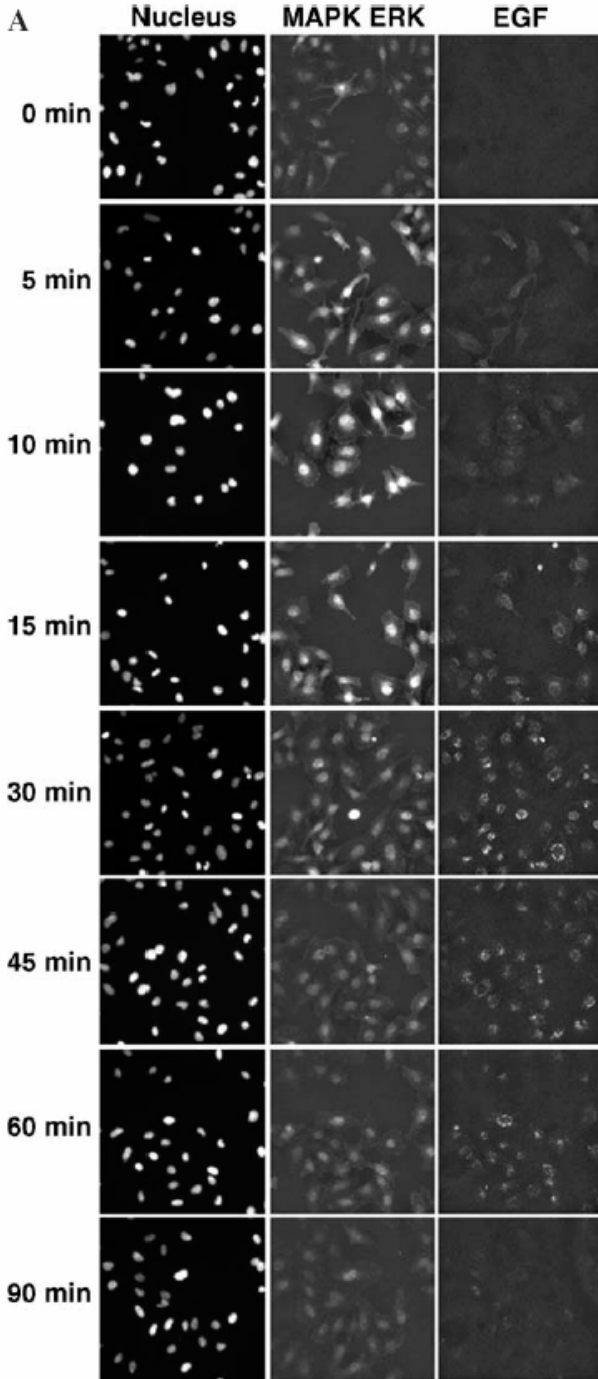


Drug Discovery Today

- ❑ Possible readouts of a translocation assay measuring the redistribution of a fluorescently labeled molecule from cytoplasm to the nucleus.
- ❑ (a) no compound effect; (b) translocation but indication of cytotoxicity (i.e. change of morphology, nuclear disintegration); (c) complete translocation; (d) compound interfering with readout (i.e. auto-fluorescence); (e) response in sub-population of cells and; (f) partial but homogeneous response.

Cell-based HCS assay to quantify the epidermal growth factor **(EGF) receptor-specific** activation of the MAPK ERK.

- The two parallel events being monitored:
 1. Phosphorylated ERK translocation to the nucleus
 2. The internalization and degradation of the EGF ligand–receptor complex in late endosomes and lysosomes initiated by EGF binding to and activating its receptor.
- Simultaneously detected, quantified, and correlated in the same cell (Compartmental Analysis BioApplication)
- Phosphorylated ERK in the nucleus is detected using indirect immunofluorescence.



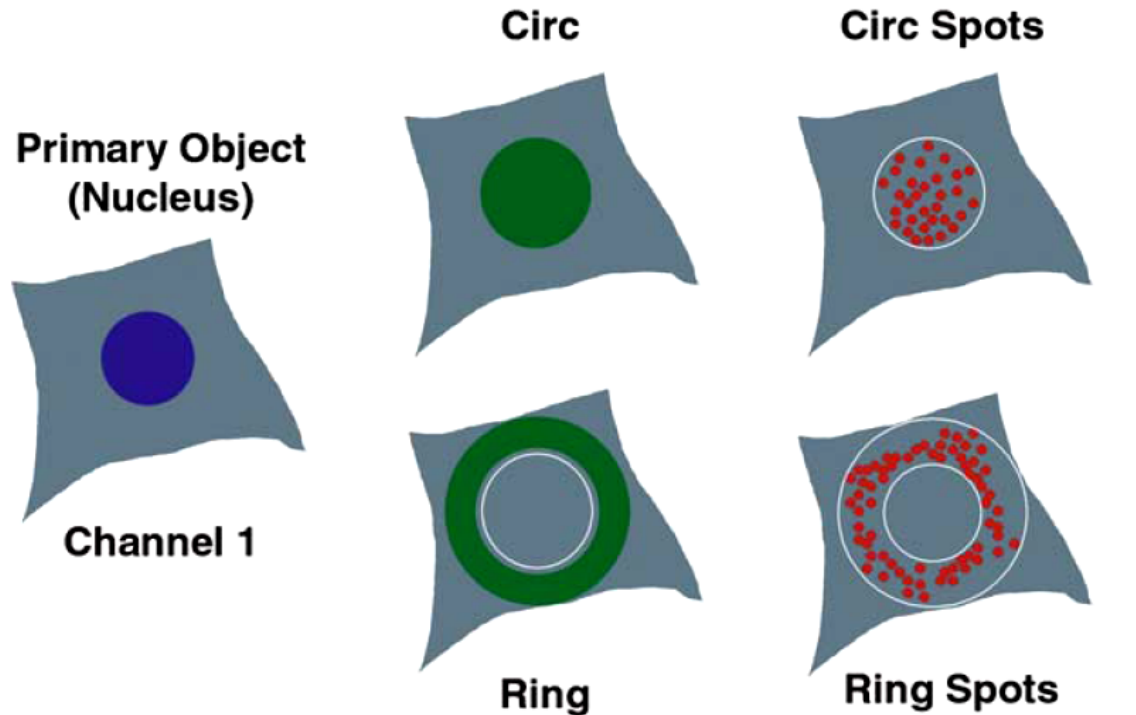
(A) Temporal sequence of fluorescent EGF internalization and ERK accumulation in the nucleus.

Procedure:

1. HeLa cells were incubated with Texas Red-conjugated EGF for the times indicated and then fixed.
2. The nuclei were labeled with Hoechst 33342,
3. The cells were then processed for immunofluorescence against ERK.
4. The sample was then imaged on the ArrayScan HCS Reader,
5. The images were analyzed with the Compartmental Analysis application.

Compartmental Analysis BioApplication

HCS Assay for EGF-Specific Activation of ERK



ERK signal in the nuclei

Internalized EGF
in endosomes and
lysosomes

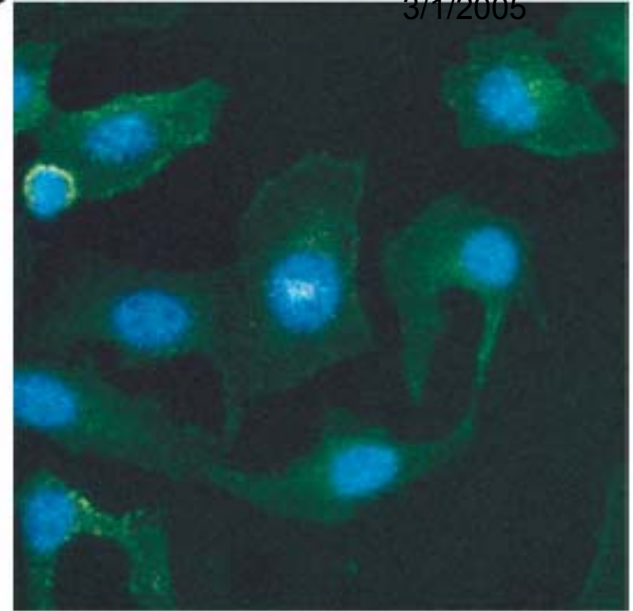
Compartmental Analysis BioApplication

- is a flexible, multi-channel,
- enables the simultaneous quantitation of the HCS Assay for presence of several different macromolecules in or between different regions or compartments in cells.
- It also can be used to simultaneously quantify the presence of several macromolecules in distinct cellular regions that are linked by the same signaling or trafficking pathway.

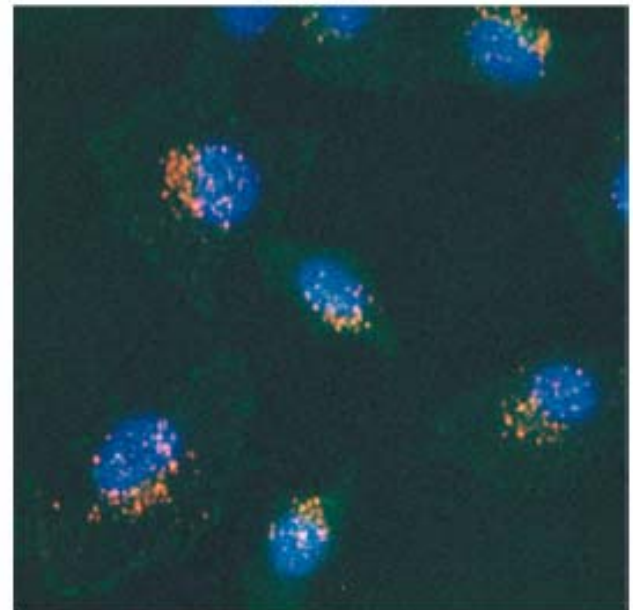
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5 min

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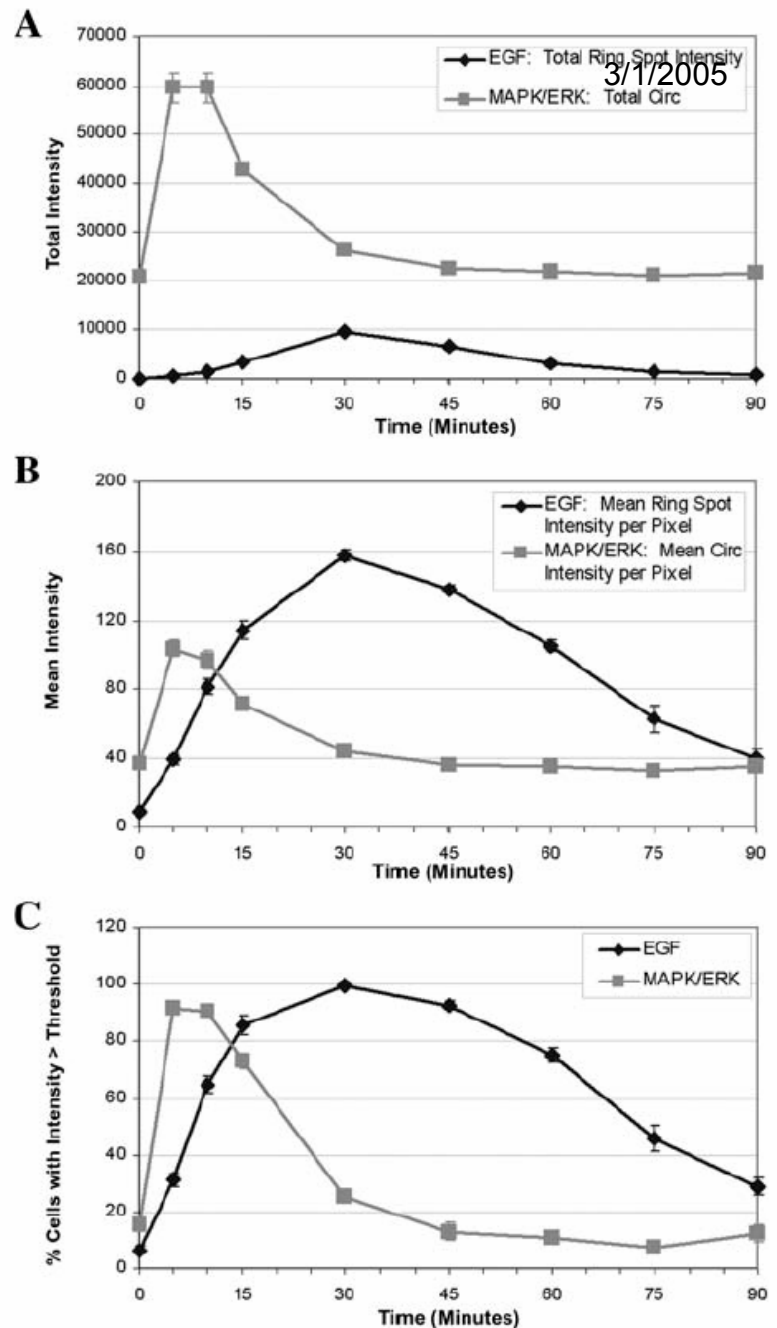
30 min

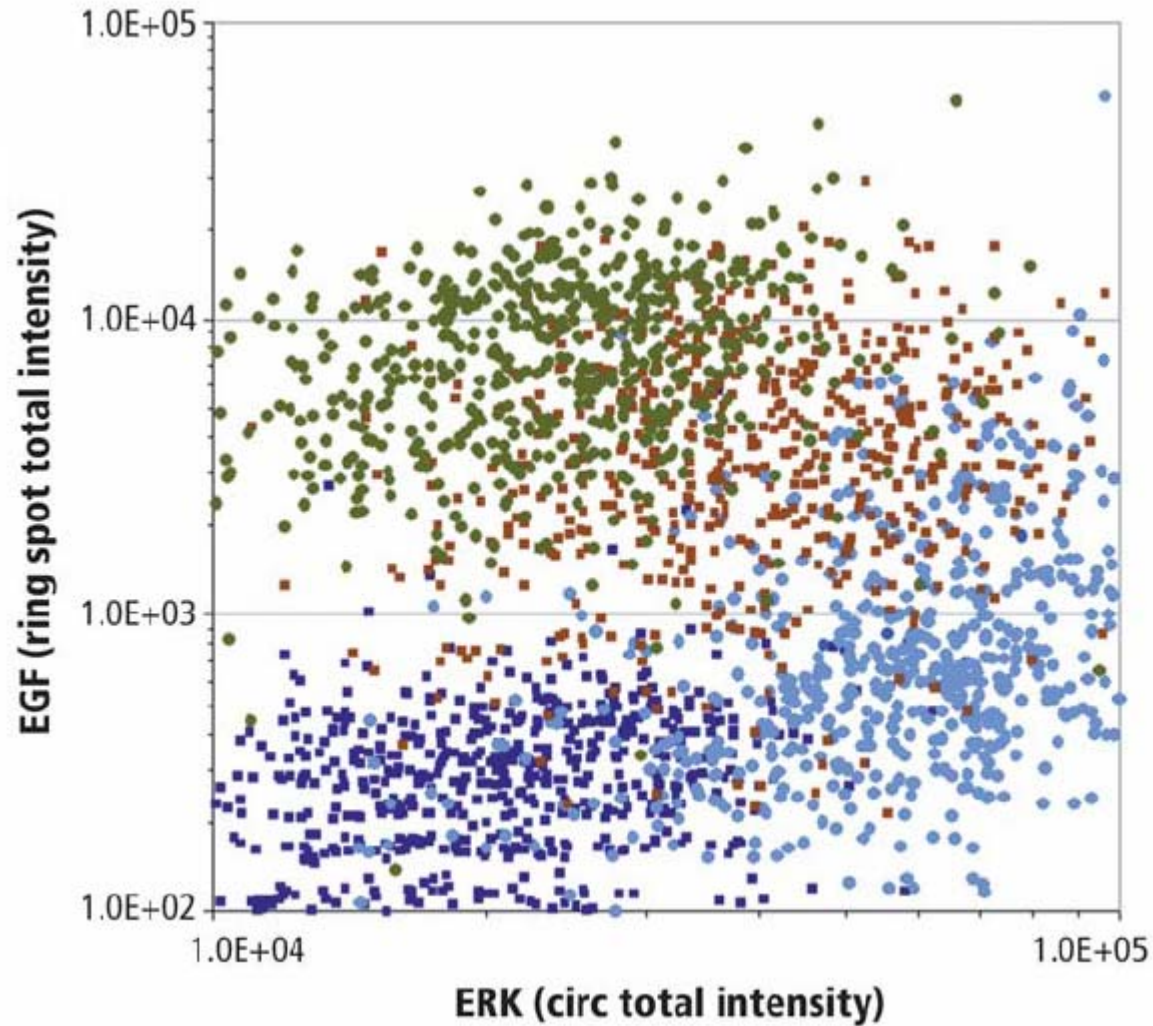


Jelena @ Wipf group

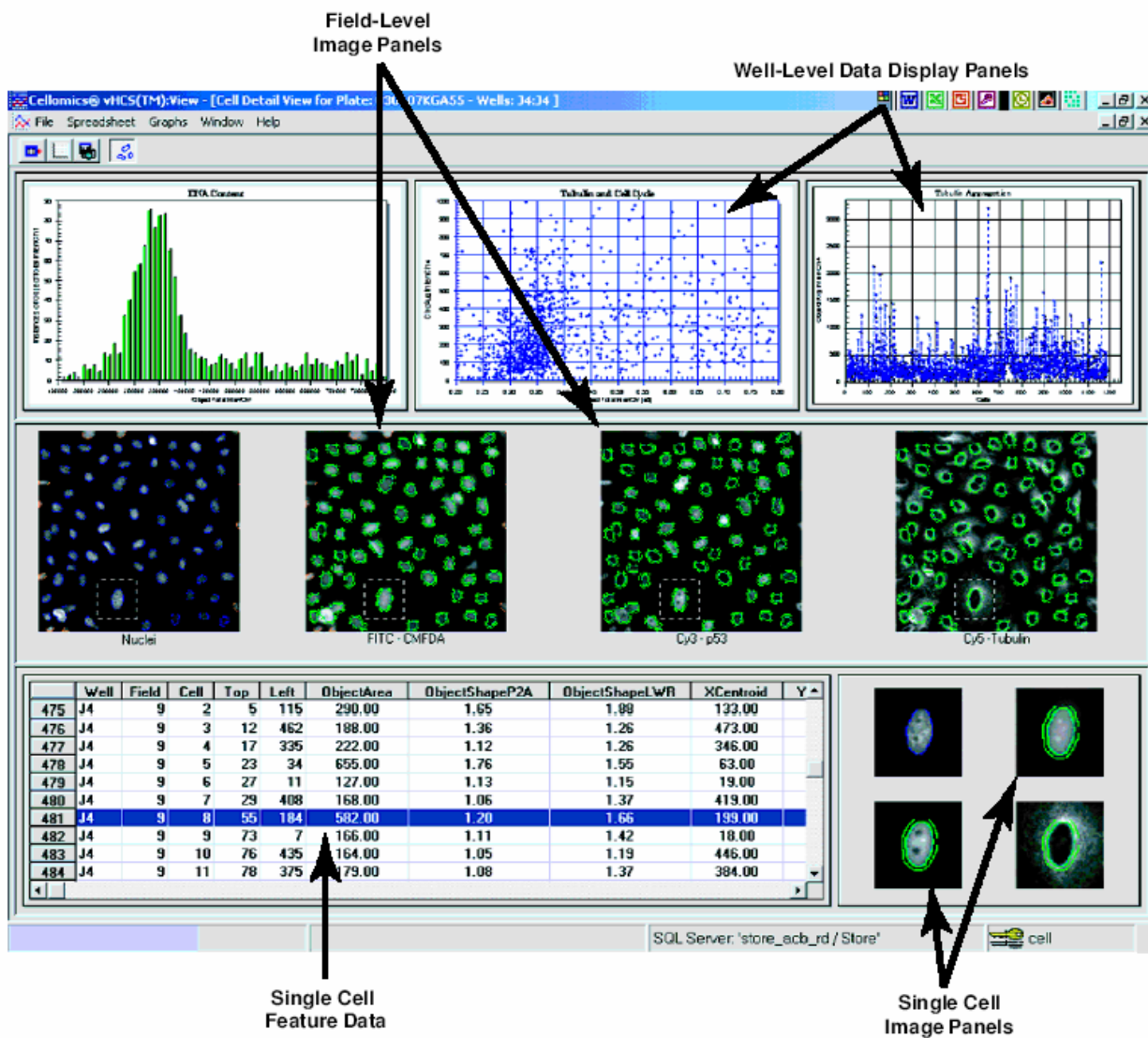
From pretty pictures to quantitative data!

- Result of analyzing images of the HeLa cells with the Compartmental Analysis BioApplication.





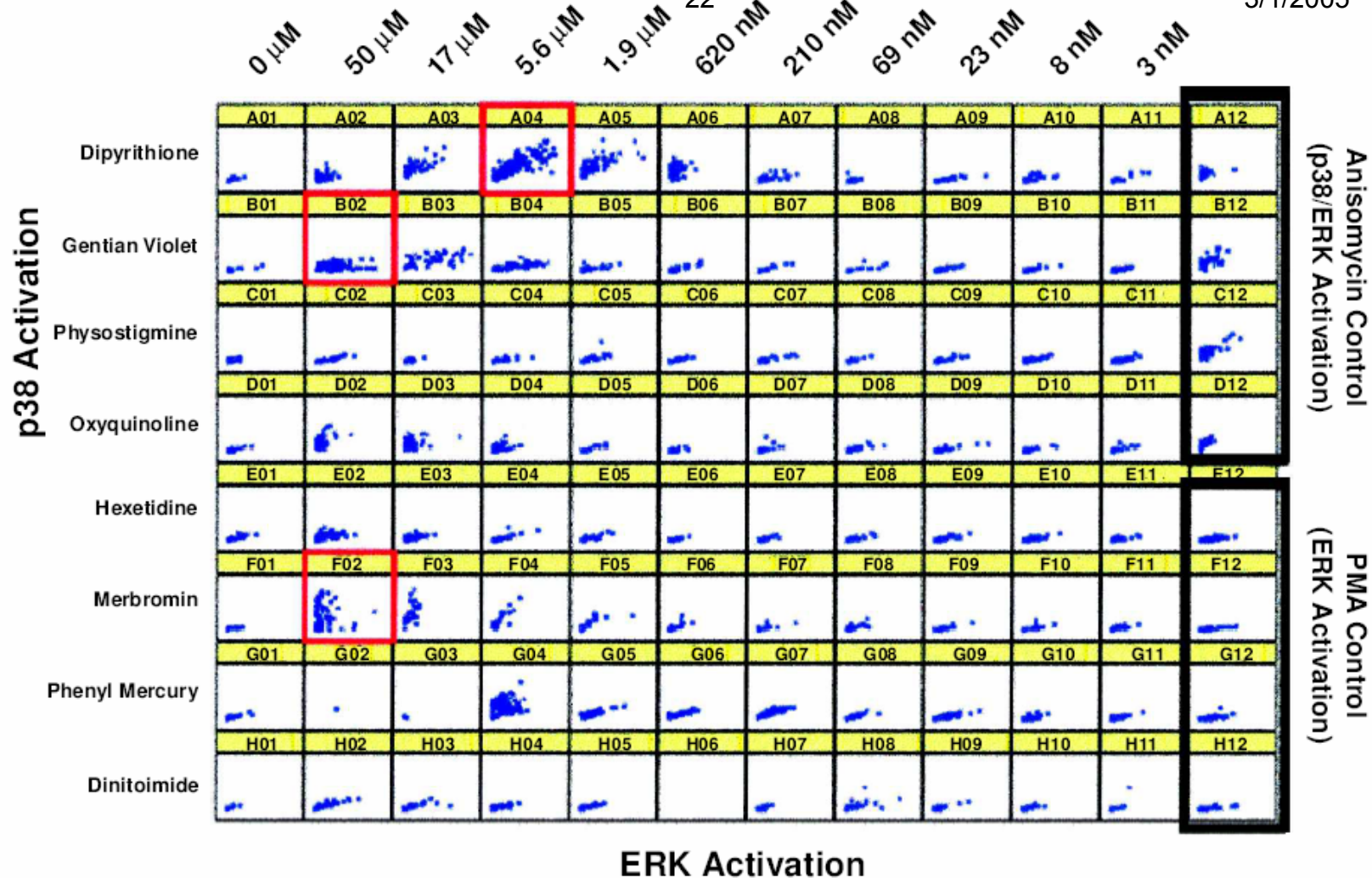
- Scatter plot showing the ERK and EGF responses for HeLa cells at different times.



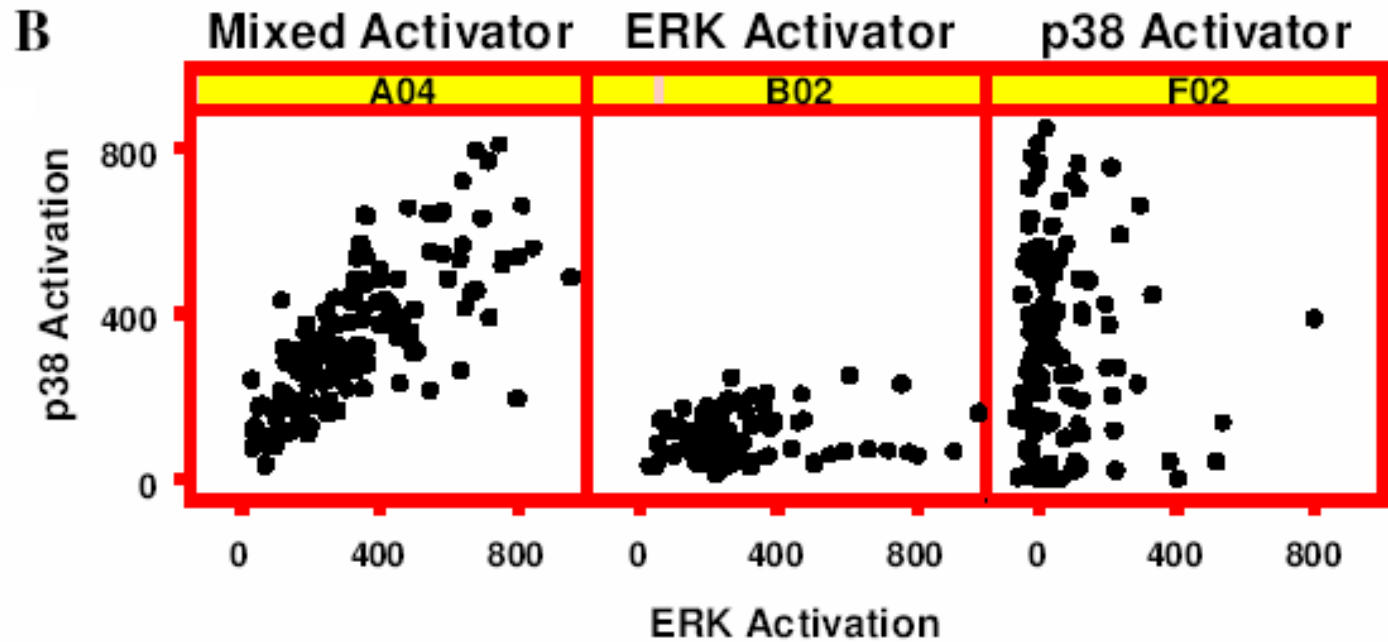
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Drug Concentration

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- Each row of well-graphs defines the concentration titration of one drug and the effect it had on the activation of two kinases.
- Each point indicates the relative activation of p38 and ERK within a single cell.



These well-graphs demonstrate how easily drilling down into HCS data can reveal complex cellular and molecular interrelationships, and the specific effects drugs have on them.

High Content Screening

