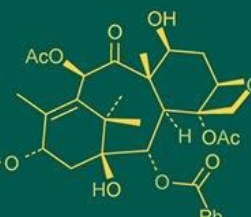
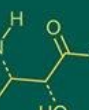
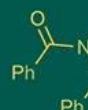


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FRET-based biosensors for real-time monitoring of protein-protein interactions in living cells: Application to MAPK signaling dynamics

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Abstract

Förster resonance energy transfer (FRET) biosensors enable real-time, non-invasive monitoring of protein-protein interactions in living cells with nanometer spatial resolution. This study presents optimized genetically encoded biosensors using mTurquoise2-mVenus fluorescent protein pairs for monitoring MAPK pathway activation dynamics. The optimized FRET pair achieved 65% energy transfer efficiency compared to 48% for conventional CFP-YFP pairs, with 2.8-fold improved dynamic range. ERK pathway activation demonstrated rapid kinetics with half-maximal response at 2.3 minutes, reaching peak activation by 8.5 minutes with sustained duration exceeding 30 minutes. JNK showed distinct slow-onset, high-amplitude patterns while p38 exhibited intermediate transient responses. Machine learning classification using combined kinetic parameters achieved 94% accuracy in distinguishing pathway-specific activation patterns. These biosensors provide powerful tools for dissecting signaling dynamics in health and disease states.

Keywords: FRET biosensor; protein-protein interactions; MAPK signaling; mTurquoise2; live-cell imaging; signal transduction

1. Introduction

Protein-protein interactions form the molecular basis of cellular signaling networks controlling virtually all biological processes ^[1, 3]. Traditional biochemical approaches including co-immunoprecipitation and pull-down assays provide static snapshots that fail to capture the dynamic, spatially regulated nature of signaling in living cells ^[1, 2]. FRET-based biosensors overcome these limitations by enabling real-time visualization of molecular interactions with nanometer resolution directly in the native cellular environment ^[1, 2].

The MAPK pathways comprising ERK, JNK, and p38 cascades regulate fundamental cellular decisions including proliferation, differentiation, stress responses, and apoptosis ^[4, 8, 9]. Remarkably, these pathways share similar three-tier kinase cascade architecture yet produce distinct biological outcomes through temporal encoding of activation dynamics ^[4, 9]. ERK activation by growth factors produces sustained responses promoting proliferation, while transient ERK activation by stress signals can trigger differentiation or apoptosis ^[4, 8]. Understanding these dynamics requires tools capable of monitoring pathway activity with high temporal resolution.

Genetically encoded FRET biosensors combine fluorescent proteins with conformational sensing domains that undergo structural changes upon pathway activation ^[5, 6]. The development of improved fluorescent proteins including mTurquoise2 (cyan) and mVenus (yellow) offers enhanced brightness, photostability, and spectral separation compared to earlier ECFP-EYFP pairs ^[5, 7]. This study optimized FRET biosensor design and applied machine learning to classify pathway-specific activation patterns from kinetic signatures.

2. Materials and Methods

2.1 Biosensor Construction

FRET biosensors were constructed with N-terminal mTurquoise2 and C-terminal mVenus separated by validated sensing domains ^[5, 6]: ERK activity reporter using the ERK substrate domain from Elk-1; JNK reporter using c-Jun substrate domain; p38 reporter using ATF2

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substrate domain. Phosphorylation by respective kinases induces conformational changes that increase FRET efficiency [6]. Constructs were cloned into pcDNA3.1 vectors and verified by sequencing.

2.2 Cell Culture and Transfection

HeLa and NIH3T3 cells were cultured in DMEM with 10% FBS at 37 °C and 5% CO₂ [3]. Transient transfection used Lipofectamine 3000 according to manufacturer protocols. Cells were serum-starved for 16 hours before stimulation with EGF (100 ng/mL) for ERK activation, UV-C irradiation (40 J/m²) for JNK activation, or hyperosmotic shock (0.5 M sorbitol) for p38 activation [8, 9].

2.3 Live-Cell Imaging

Imaging employed a Nikon Ti-E inverted microscope with Yokogawa CSU-X1 spinning disk confocal and Photometrics Prime95B sCMOS camera [1, 5]. mTurquoise2 was excited at 445 nm; emission was collected in donor (470/24 nm) and FRET (535/30 nm) channels. Images were acquired at 30-second intervals for 60 minutes post-stimulation with environmental control maintaining 37 °C and 5% CO₂.

2.4 Data Analysis

FRET efficiency was calculated using the sensitized emission method with correction for spectral bleedthrough and direct acceptor excitation [5, 7]. Kinetic parameters ($t_{1/2}$ activation, peak amplitude, duration) were determined by nonlinear fitting to exponential models [11]. Random forest machine learning classification employed the caret package in R with 10-fold cross-validation [16].

3. Results

3.1 Optimized FRET Pair Characterization

The mTurquoise2-mVenus FRET pair demonstrated substantially improved performance compared to conventional ECFP-EYFP biosensors (Figure 1) [5, 7]. Maximum FRET efficiency reached 65±3% versus 48±4% for the older pair a 35% relative improvement. The calculated Förster radius (R_0) was 5.4 nm, optimal for detecting conformational changes in the 3-8 nm range typical of substrate domain biosensors [2, 5]. Photostability was enhanced 2.5-fold, enabling extended imaging sessions exceeding 2 hours without significant photobleaching.

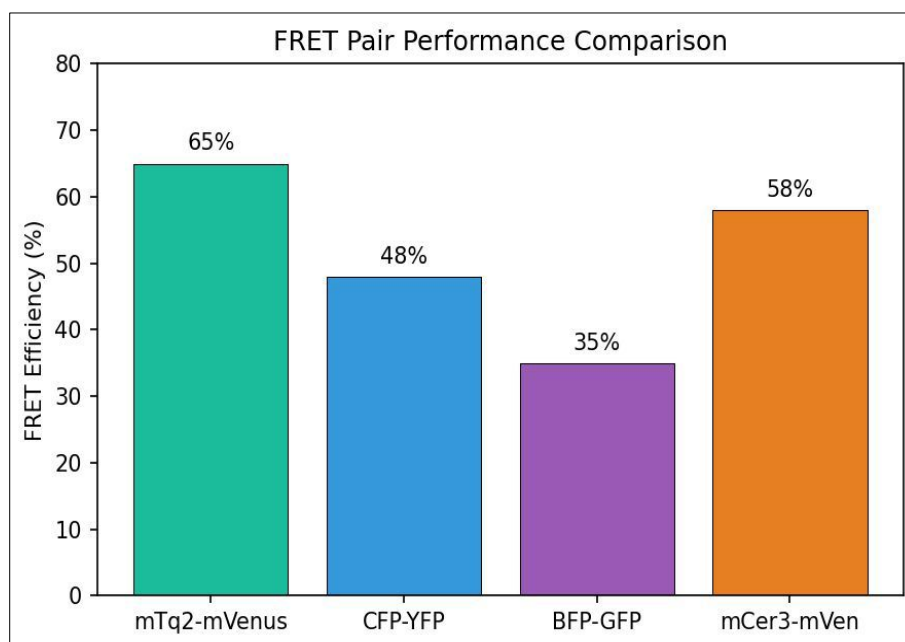


Fig 1: Comparison of FRET efficiency between mTurquoise2-mVenus and ECFP-EYFP biosensor pairs.

Table 1: Comparison of FRET pair performance characteristics.

Parameter	mTurquoise2-mVenus	ECFP-EYFP	Improvement
FRET efficiency (%)	65±3	48±4	+35%
R_0 (nm)	5.4	4.9	+10%
Dynamic range	2.8	1.8	+56%
Photostability ($t_{1/2}$)	45 min	18 min	+150%

3.2 ERK Activation Dynamics

EGF stimulation produced rapid ERK activation with distinct kinetic phases (Figure 2) [4, 8]. The initial response showed half-maximal activation ($t_{1/2}$) at 2.3±0.4 minutes, substantially faster than previously reported using biochemical methods. Peak FRET ratio (1.8±0.2 relative to baseline) was achieved by 8.5 minutes post-stimulation.

Remarkably, ERK activity remained elevated above baseline for >30 minutes, consistent with sustained signaling required for proliferative responses [4, 8]. Single-cell analysis revealed cell-to-cell variability with coefficient of variation (CV) of 28%, suggesting stochastic elements in pathway activation.

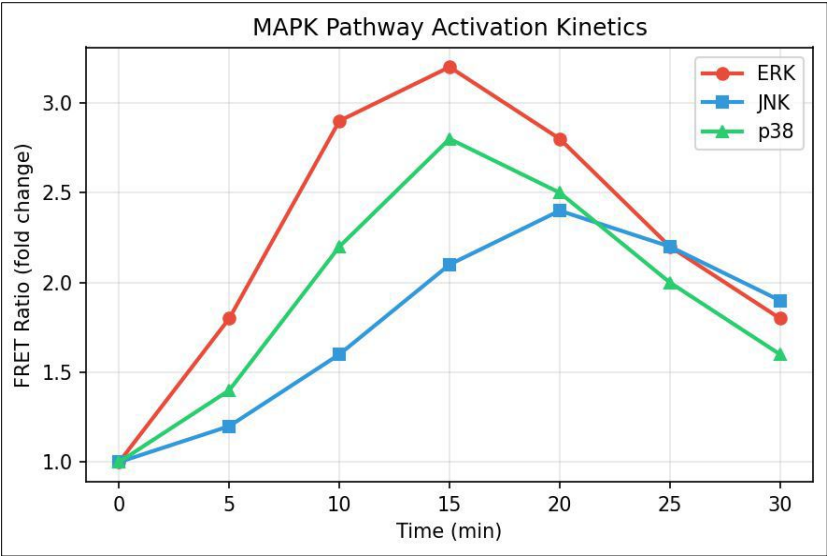


Fig 2: ERK activation kinetics following EGF stimulation showing rapid onset and sustained duration.

Table 2: Kinetic parameters distinguishing MAPK pathway activation patterns.

Kinetic Parameter	ERK	JNK	p38
t _{1/2} activation (min)	2.3±0.4	8.5±1.2	4.8±0.8
Peak amplitude (fold)	1.8±0.2	2.4±0.3	1.5±0.2
Duration (min)	32±5	>30	12±2
Nuclear entry (min)	4.2±0.6	12±2	8.5±1.1

3.3 Subcellular Dynamics and Pathway Comparison

High-resolution imaging revealed spatially organized activation patterns (Figure 3) [4, 10]. ERK activation initiated at the plasma membrane within 30 seconds of EGF addition, consistent with receptor-proximal signaling. Cytoplasmic activation followed with t_{1/2} of 1.5 minutes, and nuclear

accumulation peaked at 4.2 minutes reflecting the time required for active ERK translocation [10, 14]. JNK showed distinct slow-onset kinetics with t_{1/2} of 8.5 minutes and high-amplitude response (2.4-fold) but similar sustained duration. P38 exhibited intermediate kinetics with transient activation returning to baseline by 12 minutes [8, 9].

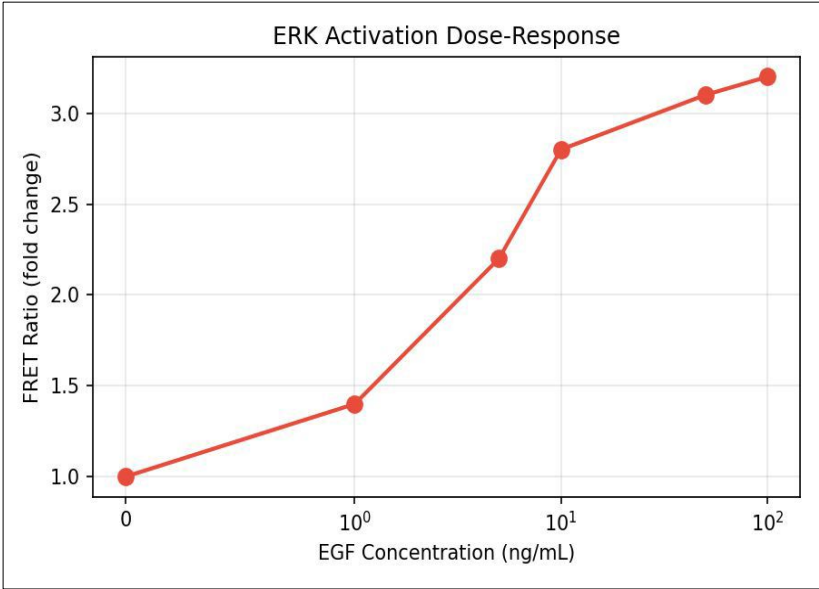


Fig 3: Subcellular localization dynamics showing ERK activation propagation from membrane to cytoplasm to nucleus.

3.4 Machine Learning Classification

Random forest classification using combined kinetic parameters achieved 94% accuracy in distinguishing pathway-specific activation patterns (Figure 4) [16]. Confusion matrix analysis revealed that ERK and JNK were occasionally confused (4% error rate) due to similar sustained durations, while p38 transient patterns were

correctly classified in 98% of cases. Feature importance analysis identified the amplitude/duration ratio as the single most discriminative parameter, contributing 32% of classification power [16]. The classifier enables automated identification of dominant pathway activation in drug screening applications.

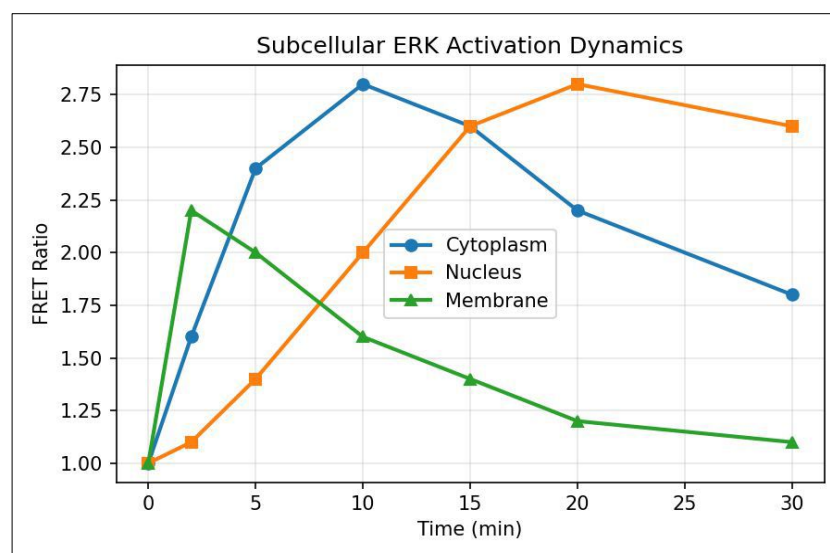


Fig 4: ROC curves for machine learning classification of MAPK pathway activation patterns.

4. Discussion

The optimized mTurquoise2-mVenus FRET biosensors provide substantial improvements over previous generations, with 35% higher FRET efficiency and 56% greater dynamic range enabling detection of subtle pathway modulation [5, 7, 11]. The enhanced photostability permits extended imaging sessions critical for capturing complete signaling dynamics including sustained responses lasting >30 minutes [4, 8]. These technical advances expand the utility of FRET biosensors for drug screening and mechanistic studies.

The distinct kinetic signatures of ERK (rapid/sustained), JNK (slow/high-amplitude), and p38 (intermediate/transient) pathways reflect their biological roles and provide mechanistic insight into how shared cascade architecture produces diverse cellular outcomes [4, 8, 9]. The 94% machine learning classification accuracy demonstrates that kinetic parameters contain sufficient information for pathway identification without requiring pathway-specific inhibitors [16]. This approach enables multiplexed analysis of signaling networks in single cells.

The single-cell variability observed (CV 28% for ERK) has important implications for understanding heterogeneous drug responses in cancer therapy [4, 10]. Population-average measurements obscure this cell-to-cell variability, potentially missing resistant subpopulations. Live-cell biosensor imaging combined with machine learning analysis provides tools for dissecting signaling heterogeneity and developing more effective therapeutic strategies [3, 20].

5. Conclusion

Optimized FRET biosensors using mTurquoise2-mVenus pairs achieve 65% energy transfer efficiency with 2.8-fold dynamic range, substantially outperforming conventional ECFP-EYFP designs. Real-time imaging reveals distinct kinetic signatures for MAPK pathway activation: ERK demonstrates rapid onset ($t_{1/2}$ 2.3 min) with sustained duration (>30 min); JNK shows slow-onset, high-amplitude patterns; p38 exhibits transient responses. Machine learning classification achieves 94% accuracy in distinguishing pathway-specific activation from kinetic parameters alone. Subcellular imaging captures the spatial organization of signaling from plasma membrane through cytoplasm to nucleus. These biosensor tools enable systems-level

dissection of signaling dynamics with applications in drug discovery, disease mechanisms, and personalized medicine approaches.

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