

## Comment on "Oscillations in NF- $\kappa$ B Signaling Control the Dynamics of Gene Expression"

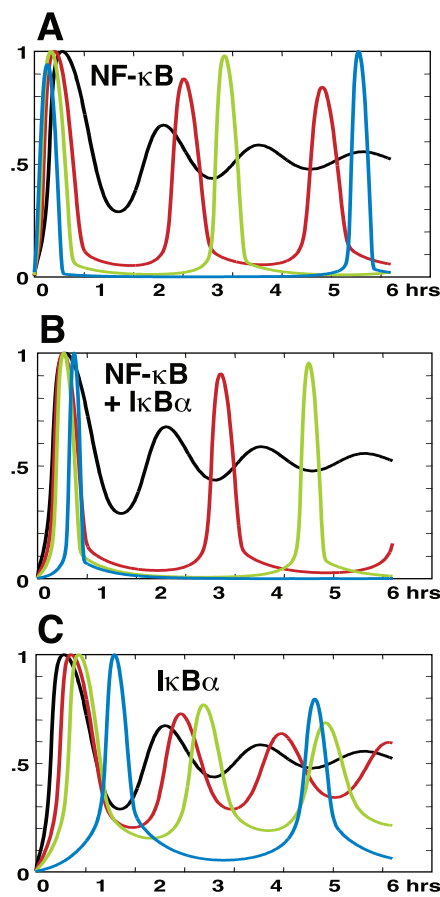
Fusing fluorescent moieties to signaling proteins of interest has allowed for the imaging of subcellular events in live cells in the hope of revealing the dynamic behavior of signal transduction networks (1–5). Nelson *et al.* (5) recently described a tour de force experimental analysis to track signaling by the transcription factor nuclear factor kappa B (NF- $\kappa$ B) in individual living cells in real time. As the authors point out, their observations match a

computational model (6) based on biochemical data derived from cell population averages. Here, we comment on interpreting experimental data derived from genetically manipulated cells and on the physiological role of sustained oscillations in NF- $\kappa$ B signal transduction.

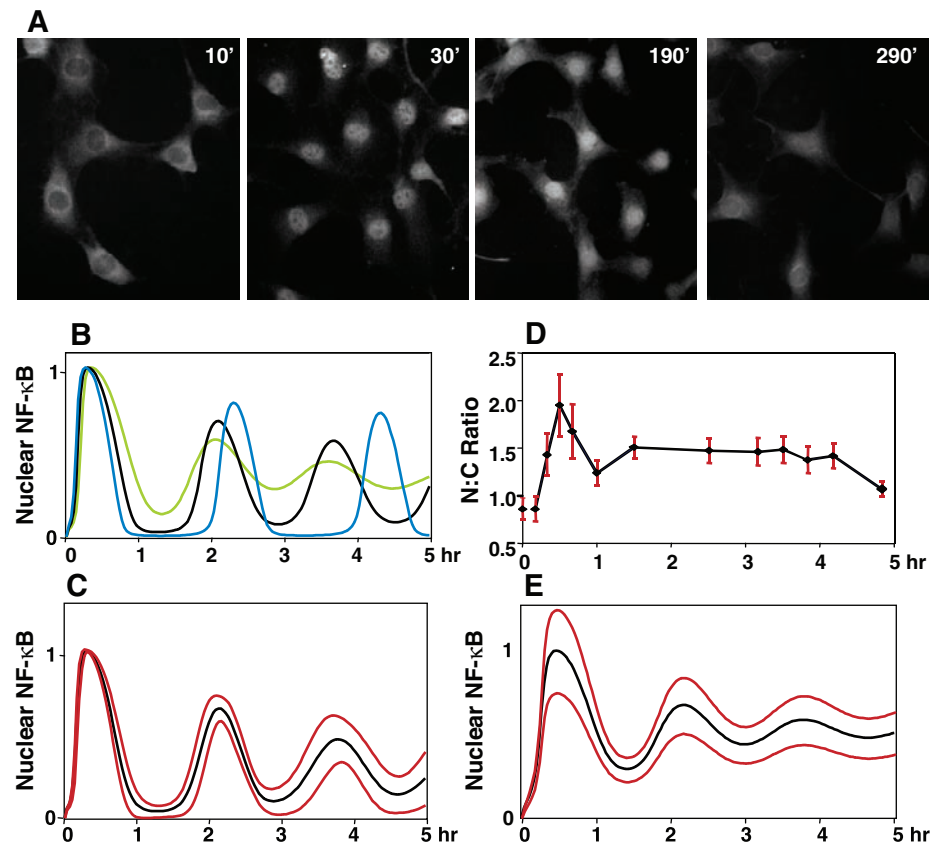
Our previously published integrated computational and biochemical analysis of NF- $\kappa$ B signaling (6) included simulations of cells with altered concentrations of NF- $\kappa$ B inhibitor proteins (I $\kappa$ B) [figure 2D in (6)]. These analyses

predicted that two I $\kappa$ B isoforms, I $\kappa$ B $\beta$  and I $\kappa$ B $\epsilon$ , have functional roles in damping the oscillatory propensity of the NF- $\kappa$ B–I $\kappa$ B $\alpha$  negative feedback loop. This was confirmed experimentally by observing damped nuclear NF- $\kappa$ B activity oscillations in wild-type cells [figures 1C and 2E in (6)] and much stronger oscillations in I $\kappa$ B $\beta$ /I $\kappa$ B $\epsilon$  double knockouts [figure 2, A and B, in (6)]. However, such cell population–based analyses may not always reveal oscillatory behavior that is occurring on the single-cell level, because protein extracts average potentially asynchronous responses of individual cells.

To study the regulation of NF- $\kappa$ B activity in real time in single cells, Nelson *et al.* (5) used ectopic expression of I $\kappa$ B $\alpha$  and NF- $\kappa$ B–p65 fused to fluorescent moieties. They transiently transfected HeLa (human cervical carcinoma) cells and SK-N-AS cells (human



**Fig. 1.** NF- $\kappa$ B activation profiles as a function of NF- $\kappa$ B protein levels. Relative (peak normalized) nuclear NF- $\kappa$ B activity graphed as a function of time in response to TNF $\alpha$  stimulation. Data derive from model simulations that reflect cells transfected with expression vectors described in (5) encoding (A) NF- $\kappa$ B–p65, (B) NF- $\kappa$ B–p65 and I $\kappa$ B $\alpha$ , and (C) I $\kappa$ B $\alpha$ . Expression of these proteins may result in protein levels that are normal (black) or increased 1.5-fold (red), 2-fold (green), or 4-fold (blue).

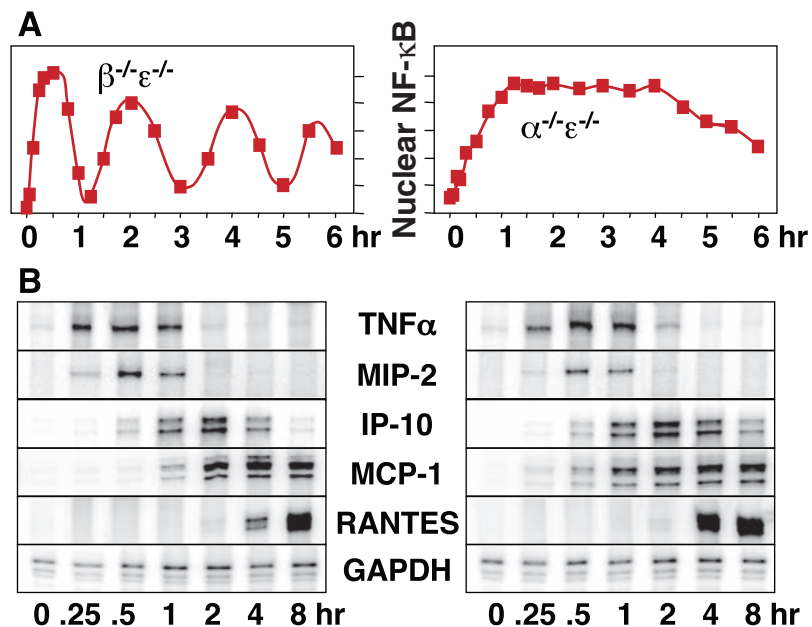


**Fig. 2.** NF- $\kappa$ B activation in single cells. (A) Immunohistochemistry for endogenous NF- $\kappa$ B in NIH 3T3 cells (mouse fibroblasts) stimulated with TNF $\alpha$ . Cells were fixed and stained at 10 min, 30 min, 190 min, and 290 min after administration of TNF $\alpha$  and stained with an antibody to p65 (9). (B) Computational simulations of oscillatory NF- $\kappa$ B in I $\kappa$ B $\beta$ / $\epsilon$ -deficient cells that contain NF- $\kappa$ B protein levels that are “wild-type” (black), 1.2x (blue), or 0.8x (green). (C) The mean NF- $\kappa$ B activity (black line) produced by computational simulations of I $\kappa$ B $\beta$ / $\epsilon$ -deficient cells containing a distribution of NF- $\kappa$ B protein levels with a standard deviation of 10%. Red lines indicate the mean  $\pm$  1 SD. (D) NF- $\kappa$ B localization data derived from immunohistological analysis graphed as nucleus:cytoplasm (N:C) ratio as a function of time in response to TNF $\alpha$  stimulation. Vertical bars indicate the standard deviation in the data for each time point. (E) Computational simulation of NF- $\kappa$ B activity in wild-type cells (6) in response to TNF $\alpha$  stimulation. Red lines indicate 1.2x and 0.8x NF- $\kappa$ B activity.

S-type neuroblastoma cells) with p65-DsRed (red fluorescent protein) and NF- $\kappa$ B-inducible I $\kappa$ B $\alpha$ -EGFP (enhanced green fluorescent protein) expression plasmids. Nelson *et al.* estimate that NF- $\kappa$ B-p65 is overexpressed 3- to 5-fold in successfully transfected cells and suggest that this has a negligible effect on the fundamental characteristics of NF- $\kappa$ B nuclear-cytoplasmic oscillations in response to stimulation by tumor necrosis factor alpha (TNF $\alpha$ ). However, model simulations (Fig. 1) predict that even 1.5- or 2-fold overexpression of either of the two components forming the negative feedback loop (NF- $\kappa$ B-p65 and I $\kappa$ B $\alpha$ , separately or in combination) can significantly alter the dynamics of NF- $\kappa$ B activity in response to TNF $\alpha$  (7, 8). First, oscillations are more persistent because the damping effects mediated by I $\kappa$ B $\beta$  and I $\kappa$ B $\epsilon$  are diminished relative to the negative feedback effects mediated by both overexpressed NF- $\kappa$ B and inducible I $\kappa$ B $\alpha$ . This situation is analogous to the relative strengthening of the negative feedback by knocking out nonfeedback I $\kappa$ B $\beta$  and I $\kappa$ B $\epsilon$  isoforms (6). Second, the oscillation frequency may dramatically change, which suggests that cells with different degrees of overexpression may have oscillatory responses with drastically different periods. We suggest that as a result of inevitable cell-to-cell variations in the amount of plasmid DNA, transfected cells are likely to be much less synchronous in their NF- $\kappa$ B response than are untransfected cells.

Nelson *et al.* (5) confirm that the negative feedback loop of NF- $\kappa$ B-p65 and I $\kappa$ B $\alpha$  has a strong propensity for oscillations and that such dynamic behavior can be studied productively with computational models. In our view, their results also caution about interpreting the dynamic recordings of cells genetically manipulated with GFP fusion proteins: Oscillations recorded in overexpressed feedback systems do not allow us to conclude that oscillations of the same persistence, amplitude, and period occur in normal, genetically unaltered cells. GFP experiments may be more conclusive when clonal cell lines are established in which individual cells are genetically identical and expression levels of the exogenous fusion proteins and functionally relevant endogenous signaling proteins can be quantitated to inform computational simulations.

An alternative way to experimentally address the response in single cells is to perform immunohistochemical analysis of individual, but genetically unmodified, cells (Fig. 2A). This approach does not allow tracking of individual cells over time, but can reveal the variance of responses across the cell population at different time points. Variance in measured data may be the result of technical variation (error in measurement) or biological variation, (e.g., due to asynchrony). If the



**Fig. 3.** Target-gene expression in response to different NF- $\kappa$ B activity profiles. (A) NF- $\kappa$ B activity profiles in response to persistent TNF $\alpha$  stimulation in I $\kappa$ B $\beta$ <sup>-/-</sup>ε<sup>-/-</sup> (left) and I $\kappa$ B $\alpha$ <sup>-/-</sup>ε<sup>-/-</sup> (right) murine embryonic fibroblasts, as measured in (6). (B) mRNAs of known NF- $\kappa$ B response genes (10) were measured by RNase protection assay at the indicated times during persistent TNF $\alpha$  stimulation.

responses of individual cells are asynchronous and show undamped oscillations (Fig. 2B), one would expect higher variance at late times than at early times and lower averages due to prolonged phases between peaks (Fig. 2C). However, we consistently found that the variance of the responses is lower at late times than at early times and that averages remain high (Fig. 2D). Such a variance distribution is more consistent with a simulation in which the observed variance is derived from technical measurement errors that may be assumed to be proportional to the measured value itself (Fig. 2E). These data suggest that NF- $\kappa$ B activation in genetically unmodified fibroblasts is synchronous and highly damped.

The regulation of NF- $\kappa$ B may vary greatly between different cell types, and it is often misregulated in disease-associated cells. Persistent oscillations might occur in some of the many possible physiological or pathological scenarios, even if they have not been observed in the genetically unaltered cells examined so far. Nelson *et al.* show that persistently oscillatory NF- $\kappa$ B activity leads to a higher expression of an exogenous reporter gene than a single transient pulse of NF- $\kappa$ B activity [figure 2, G to J, in (5)]. Similarly, we showed that sustained activity allows for quantitative and qualitative changes in the expression of some endogenous genes [figure 4, B and C, in (6)]. To investigate whether persistent oscillations of NF- $\kappa$ B could play a physiological role in gene expression, persistently oscillating NF- $\kappa$ B should be compared with sustained steady NF- $\kappa$ B activity. To determine whether oscillations are impor-

tant in NF- $\kappa$ B functionality, we examined the expression of NF- $\kappa$ B target genes in cells harboring a single I $\kappa$ B isoform that either provides for negative feedback-induced oscillations or does not (Fig. 3). Our results do not reveal marked differences in resultant gene expression, and mice deficient in the nonfeedback I $\kappa$ B isoforms do not present strong phenotypes (data not shown). Thus, it remains unclear whether persistent oscillations of NF- $\kappa$ B activity regulate gene expression qualitatively, quantitatively, or in dynamics.

In summary, we suggest that perturbations of genetic circuits with ectopic expression of fluorescently labeled proteins should be interpreted with caution, as significant deviations from normal cellular signaling characteristics might result. Although oscillatory behavior of endogenous proteins in cell cycle and circadian regulatory mechanisms is well documented, it remains unclear whether the propensity for oscillations in cytokine or stress response signaling is functionally and physiologically important or is merely a consequence of post-induction repression mechanisms that allow for rapid adaptation after productive transduction of signals.

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7. Simulations were done with the computational model described in (6) and also used in (5). Parameters values were as described, with the exception that the NF- $\kappa$ B concentration and the parameters tr2 and tr2a (accounting for the rates of constitutive and NF- $\kappa$ B-induced transcription) were increased as

shown in the Fig. 1 legend to account for over-expression effects due to transfected plasmids.

8. Simulations of the NF- $\kappa$ B response in I $\kappa$ B $\beta$ / $\epsilon$ -deficient cells were done as described (6), with tr2b and tr2e parameters set to zero. Starting amounts of NF- $\kappa$ B were as stated in the legends or were varied according to a normal distribution with a standard deviation of 10%. Mean and standard deviation of the NF- $\kappa$ B activity normalized to the maximum were calculated for the time course and graphed.
9. Nuclear and cytoplasmic fluorescence intensities of individual cells were measured using IPLab; background of each captured fluorescence image was subtracted out. The Hoechst 33342 stained nucleus was used to determine the nuclear region by IPLab's Autosegment function. Nuclear segments were eroded twice to avoid contamination from cytoplasmic regions. Representative cytoplasmic regions were selected by taking the boundary pixels of the on-dilated nuclear segment (to minimize nuclear overlap). Relative fluorescence was calculated by taking the ratio of the mean intensity of the nuclear region to that of the cytoplasmic region. Forty to 100 cells were analyzed for each time point to assure statistical significance.
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