

# An Integrated Chemical Cytometry Method: Shining a Light on Akt Activity in Single Cells

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**Abstract:** Tools to evaluate oncogenic kinase activity in small clinical samples have the power to guide precision medicine in oncology. Existing platforms have demonstrated impressive insights into the activity of protein kinases, but these technologies are unsuitable for the study of kinase behavior in large numbers of primary human cells. To address these limitations, we developed an integrated analysis system that utilizes a light-programmable, cell-permeable reporter deliverable simultaneously to many cells. The reporter's ability to act as a substrate for Akt, a key oncogenic kinase, was masked by a 2-4,5-dimethoxy 2-nitrobenzyl (DMNB) moiety. Upon exposure to ultraviolet light and release of the masking moiety, the substrate sequence enabled programmable reaction times within the cell cytoplasm. When coupled to automated single-cell capillary electrophoresis, statistically significant numbers of primary human cells were readily evaluated for Akt activity.

The appropriate prescription of targeted therapeutics for cancer treatment relies on the accurate identification of aberrant signaling pathways, most frequently those involving protein kinases. However, the selection of patients for pathway inhibition is mostly empirical or reliant upon sequencing to identify mutations within the genome. A targeted selection process that reveals those patients whose cells possess increased kinase activity would lessen costs as well as forego drug toxicity in individuals not likely to be responsive to pathway inhibition. Thus, kinase-activity sensors and their attendant analytical platforms to identify aberrant kinase activities in primary cells independent of their genomic mutation status would be of high value.<sup>[1]</sup> However,

the very small sample size of most clinical specimens and limited throughput of analysis has precluded the routine measurement of kinase activity in single human primary cells.<sup>[2]</sup> The requirements for these measurements are two-fold: 1) a sensor that is selective for the kinase of interest, deliverable simultaneously to single cells, and resistant to phosphorylation until the desired assay start time, and 2) a platform that can analyze statistically relevant numbers of single cells.

Fluorescent peptide sequences are utilized as reporters to directly assay kinase activity and stand to bridge the gap between small molecules, which are poor substrates of limited specificity, and genetically engineered proteins, which are difficult to transfect uniformly into cells.<sup>[3]</sup> Peptide reporters phosphorylated by kinases within cells are highly sensitive, quantitative, and possess great multiplexing potential when coupled to capillary electrophoresis (CE). To date, the delivery of these reporters into the cell has relied on low-throughput methods, such as microinjection, or on cell-penetrating peptides (CPPs), which largely deliver cargo to endosomal compartments.<sup>[4]</sup> Additionally, reporters delivered by CPPs may be acted upon by the kinase of interest during the delivery process, thus the reaction time in the cell is unknown. Delivering a reporter into the cytosol of large numbers of cells while retaining control of assay initiation would dramatically improve overall throughput when coupled to an automated single-cell assay platform. The latter would enable sampling from sufficient numbers of cells for statistical analyses and identification of cellular subpopulations.

We addressed these opportunities by integrating chemical and instrumental innovations. Specifically, we have developed a cell-permeable, photoactivatable reporter that is chemically masked (Scheme 1) and integrated into an automated analysis platform. The active reporter acts as a substrate for the protein kinase Akt, a kinase which plays critical roles in tumor formation and progression.<sup>[5]</sup> The native substrate (NS) Akt peptide reporter 6FAM-GRP-(R)-AFTF-(A)-Amide (R and A represent *N*-methylated versions of amino acids) has favorable phosphorylation kinetics, specificity, and a demonstrated resistance to intracellular degradation.<sup>[6]</sup>

NS possesses a single hydroxy group that, when coupled to a photoremovable DMNB group, should block phosphorylation by Akt and thus silence the reporter as a substrate (Figure 1 b). Figure 1 a and the Supporting Information, Figure S1 demonstrate the photochemical conversion of inactive to active reporter. Importantly, the active reporter **2** is phosphorylated at a rate indistinguishable from that of NS, both in vitro and in cell lysates (Figure 1 b). The inactive Akt reporter **1** is not phosphorylated nor spontaneously converted

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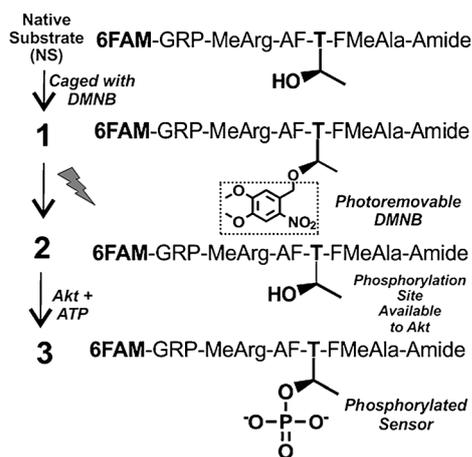
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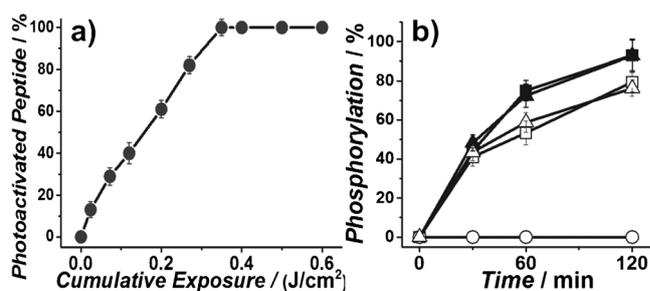
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**Scheme 1.** Native substrate (NS) was chemically caged with DMNB to generate **1**, which is delivered intracellularly. Photolysis releases the active sensor **2** (identical to NS), which is phosphorylated (**3**) and measured in single cells by automated single-cell capillary electrophoresis.

to its active counterpart in the dark during the assays (Supporting Information, Figure S6).

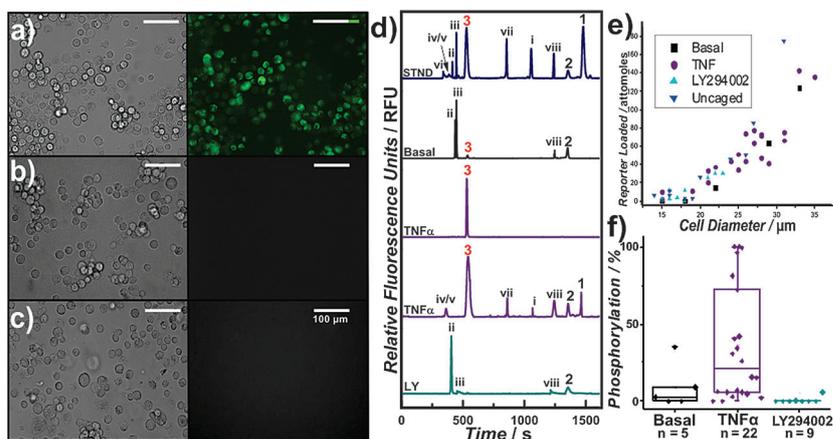
Having demonstrated that **1** is photoactivated and subsequently phosphorylated by Akt, we explored the delivery options for this reporter. Since NS is excluded from PANC-1 cells, prior work required microinjection of the reporter into cells, which is a protracted process. Many CPPs have been reported, but most are taken up through endosomal pathways, resulting in significant peptide degradation and little to no delivery to the cytosol, which is the primary location of Akt.<sup>[7]</sup> These challenges were manifested in our initial attempts to render NS cell-permeable, with multiple CPPs<sup>[8]</sup> appended to the reporter resulting in a punctate distribution of the reporter after uptake into the cell, which is indicative of a non-cytoplasmic location (Supporting Information, Figure S4). However, NS, when modified with DMNB (**1**), is sufficiently hydrophobic to enter into the cytosol when incubated with PANC-1 cells (Figure 2a and the Supporting Information, Figure S5). The diffuse (rather than punctate) appearance of the fluorescence after loading peptide into the cells (Supporting Information, Figure S5) combined with the observed phosphorylation of the reporter (post photoactivation) (Figure 2d) suggests delivery into the cytosol rather than a cytosolic subcompartment. Quantification of the amount of intracellular peptide by CE revealed a correlation ( $R^2 = 0.79$ ) between the total moles of fluorescent reporter ( $n_{\text{total}} = n_{\text{caged}} + n_{\text{phosphorylated}} + n_{\text{degraded}}$ ) and the cell diameter (Figure 2e). NS modified with a DMNB-Ser moiety (rather than DMNB-Thr) is likewise cell-permeable (Supporting Information, Figure S7), sug-



**Figure 1.** In vitro characterization of caged AKT reporter. a) Photolysis of **1** (10  $\mu\text{M}$ ) in extracellular buffer (ECB). b) Phosphorylation assays with recombinant Akt. In vitro assays in buffer were performed with peptides NS ( $\blacksquare$ ), **1** ( $\bullet$ ), and **2** ( $\blacktriangle$ ). Experiments in PANC-1 lysates were performed with peptides NS ( $\square$ ), **1** ( $\circ$ ), and **2** ( $\triangle$ ) (Supporting Information, Section S3.5).

gesting that the addition of DMNB may be sufficient to render peptides of similar hydrophobicity cell-permeant.

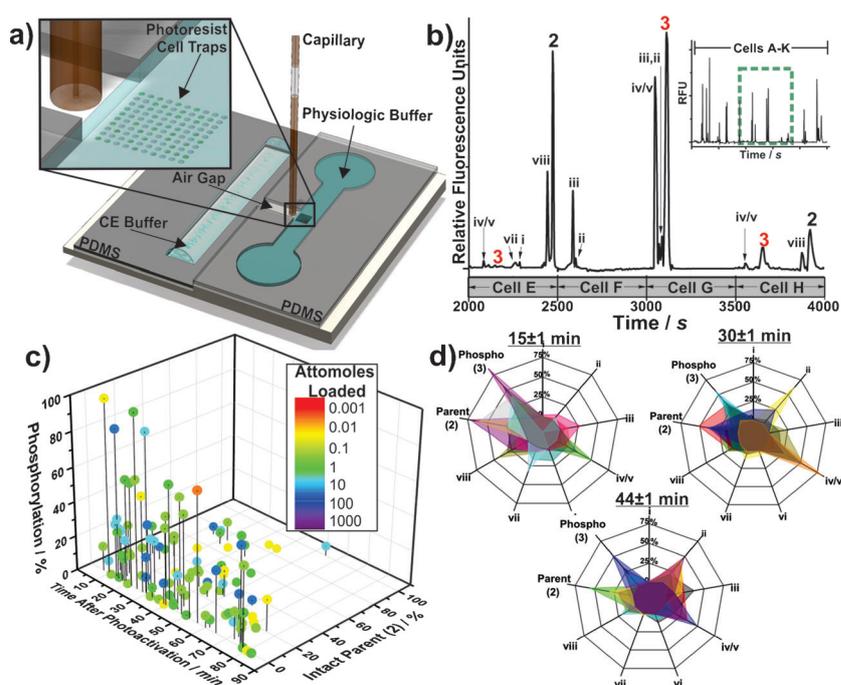
We next evaluated the ability of this reporter to detect Akt activation after photolysis within intact PANC-1 cells. Akt is upstream of DNA repair mechanisms and is known to be a stress-activated kinase. Thus, we first assessed whether the UV exposure needed for photoactivation might cause DNA damage and/or activate Akt. CE-analysis revealed that UV illumination ( $0.4 \text{ J cm}^{-2}$ ) of cells transforms  $96 \pm 2.2\%$  of **1** into active **2**. UV exposure under these conditions does not induce cytotoxicity or DNA pyrimidine lesions (Supporting Information, Figures S8 and S9). Using these conditions, we assessed Akt activation and inhibition in single PANC-1 cells (Figure 2 d,f). After photolysis, the contents of the single cells were assayed by CE and the phosphorylated sensor was quantified. Without endogenous stimuli, minimal Akt activity



**Figure 2.** Intracellular characterization of peptide **1**. Images of cells incubated at  $4^\circ\text{C}$  with a) **1**, b) **2**, and c) without peptide. d) Single-cell Akt activity measured by chemical cytometry with the electrophoretic peaks labeled as **1**, **2**, and **3** as denoted in Scheme 1. 6FAM-labeled proteolytic products of **1** are labeled as i–viii (Supporting Information, Table S2). The STND trace shows the migration time of a standard solution of the peptides. Traces below are from individual PANC-1 cells that were deprived of serum (basal), stimulated with  $100 \text{ ng mL}^{-1}$  TNF $\alpha$ , or treated with  $10 \mu\text{M}$  LY294002 +  $100 \text{ ng mL}^{-1}$  TNF $\alpha$ . e) The amount (in attomoles) of reporter loaded as a function of cell diameter ( $R^2 = 0.79$ ). f) Akt activity for a population of single cells analyzed 20 min post-photolysis. Prior to loading with **1**, photolysis, and analysis, the cells were treated as described in (d).

was detected (Figure 2f), which suggests that the illumination protocol does not activate Akt.<sup>[5]</sup> We also examined Akt pathway stimulation and inhibition in response to TNF $\alpha$  and TNF $\alpha$  + LY294002, respectively. Significant heterogeneity in reporter phosphorylation in response to TNF $\alpha$  stimulation (0–100%) was observed, consistent with reports of mosaic Akt activation in individual cells of a cell population.<sup>[6,9]</sup> The PI3K inhibitor, LY294002, blocked reporter phosphorylation in response to TNF $\alpha$ , as expected since PI3K is downstream of the TNF $\alpha$  receptor and upstream of Akt. Having produced a cell-permeable and photoactivatable Akt reporter, we sought to harness these unique properties to enhance the throughput of single-cell Akt activity measurements. Previous work describing the microinjection of peptide-based protein kinase sensors offered a very limited throughput of 0.5 cell h<sup>-1</sup>, which is not biomedically useful.<sup>[6]</sup> A recently reported automated single-cell CE system quantified kinase activity as rapidly as 3.5 cells min<sup>-1</sup>, rivaling microfluidic single-cell electrophoresis systems and offering excellent (10<sup>-20</sup> mol) detection limits.<sup>[10]</sup> A limitation of the automated system, however, is that reporters are active substrates during the loading process into the cell, so that the reaction time in the cell is unknown. These challenges are uniquely addressed by reporter **1**, which is membrane-permeant with a programmable light-triggered reaction initiation.

We conducted a pilot study in which the unique properties of compound **1** were combined with the automated single-cell CE system. All cells in a population were simultaneously photo-activated to assess Akt activity as a function of time. Cells were loaded with compound **1**, illuminated, and serially analyzed ( $n = 109$ ) (Figure 3). The photoactivation efficiency is  $97 \pm 2.8\%$  within intact cells, with  $42.4 \pm 42.7$  amol of the reporter loaded per cell. The amount of reporter (amol) detected correlated with the cell volume (Figure 2e) and appeared to be independent of the dwell time ( $R^2 = 0.018$ ; Supporting Information, Figure S10), which suggests that the reporter is not exported by the cell over the analysis period. An oft-overlooked commonality among peptides, even when engineered for stability, is their eventual degradation within the intracellular environment. Phosphorylation, degradation, and other intracellular alterations or environments can have similar effects on sensor fluorescence properties, creating a challenge for many sensors relying solely on fluorescence properties as a proxy for sensor phosphorylation.<sup>[11]</sup> In contrast, the single-cell CE system measures intact and degraded reporter as well as phosphorylated reporter to accurately quantify the phosphorylation rate (Figure 3b). When peptide standards of known concentration are also



**Figure 3.** Automated single-cell analysis. a) The microfabricated integrated system used for automated single-cell CE analysis. Cell traps (45  $\mu\text{m}$  deep, inset) were loaded with PANC-1 cells and the peptide within the cell was photoactivated. Cells were then assayed. b) Separation of intracellular contents from consecutively analyzed cells. Trace shows peaks for **2**, **3**, and proteolytic products of **1** (i–viii) for four single cells (E–H). Inset: Trace showing the separated contents of 11 cells (A–K). c) Reporter metabolism in PANC-1 cells. Each colored data point represents a single cell with the color denoting the amount of reporter (in moles) loaded into the cell. d) Reporter metabolism at various time points after photoactivation. Each overlay (color) represents the metabolism from a single cell.

paired with single-cell CE, the amount of the different peptide species are readily quantified (Figure 3c).

Rather than simply increasing as a function of reporter incubation time, the rate of Akt reporter phosphorylation in single cells was highly variable ( $0.0\text{--}0.024$  zmol pg<sup>-1</sup> s<sup>-1</sup>) at all times measured (Figure 3c the Supporting Information, Figure S9). While we and others have demonstrated heterogeneous Akt activity in PANC-1 and other cell lines at individual endpoints,<sup>[6,9,12]</sup> reporter **1** enabled time-resolved studies of this heterogeneity, suggesting that peptide phosphorylation may reach an equilibrium determined by the balance of kinase and opposing phosphatase activity, with a unique equilibrium point for each cell (Supporting Information, Figure S11). As Akt is a major promoter of cell survival,<sup>[13]</sup> this type of heterogeneity has been suggested to promote survival of a subset of cells under stressful conditions and/or confer resistance to chemotherapeutics.<sup>[14]</sup> The integrated analysis system also delivers insight into how peptides are modified over time within single cells (Figure 3d; fragment naming in the Supporting Information, Table S2), and the formation of key fragments can be monitored (Supporting Information, Section S4).

The cell-permeable, photoactivated reporter, combined with an integrated platform, enabled the analysis (7.2 cells h<sup>-1</sup>) of kinase activity in a population of cells at a substantially higher throughput than previous studies. The



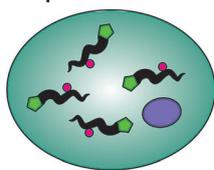
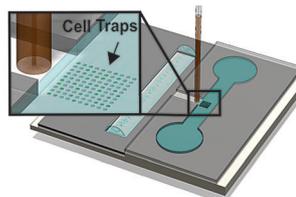
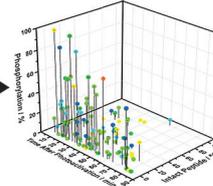
## Communications



## Biosensors

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An Integrated Chemical Cytometry  
Method: Shining a Light on Akt Activity in  
Single Cells

Photoactivated  
Peptide BiosensorAutomated Analysis  
PlatformKinase Activity  
Profiles

**See the light:** An integrated analysis system that utilizes a light-programmable, cell-permeable reporter that can be delivered simultaneously into many cells was developed. The reporter is a sub-

strate for Akt, a key oncogenic kinase, and was used to evaluate the Akt activity of statistically significant numbers of primary human cells through automated single-cell capillary electrophoresis.