Isoelectric focusing technology quantifies protein signaling in 25 cells

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A previously undescribed isoelectric focusing technology allows cell signaling to be quantitatively assessed in <25 cells. High-resolution capillary isoelectric focusing allows isoforms and individual phosphorylation forms to be resolved, often to baseline, in a 400-nl capillary. Key to the method is photochemical capture of the resolved protein forms. Once immobilized, the proteins can be probed with specific antibodies flowed through the capillary. Antibodies bound to their targets are detected by chemiluminescence. Because chemiluminescent substrates are flowed through the capillary during detection, localized substrate depletion is overcome, giving excellent linearity of response across several orders of magnitude. By analyzing pan-specific antibody signals from individual resolved forms of a protein, each of these can be quantified, without the problems associated with using multiple antibodies with different binding avidities to detect individual protein forms.

cell signaling | immunoassay | phosphorylation | Western blot | microfluidic

Cell signaling often is achieved by the phosphorylation and consequent activation of proteins, especially kinases (1, 2). Most methods used to detect phosphorylation of signaling molecules use phosphorylation-specific antibodies that selectively bind to individual phosphorylated sites. Such antibodies are used in, for example, Western blotting (3, 4), ELISAs (5, 6), dot blots (7), protein arrays (8), FACS (9) and fluorescence microscopy (10). There are two principal limitations of such methods. First, different antibodies are typically used to detect the protein of interest and its various phosphorylated forms. Necessarily, each of these antibodies will exhibit different binding avidity for their target epitope. This is problematic for quantitatively assessing phosphorylation (and therefore activation) of the protein. Typically, only relative phosphorylation (comparing one sample to another) can be reported in such studies. The second principal limitation is that for many phophorylation sites, specific antibodies simply are not available.

Methods used to analyze cell signaling by protein phosphorylation include those antibody-dependent techniques referenced above, as well as methods not using antibodies, such as mass spectrometry (11, 12). Although recent advances in such techniques are impressive, most suffer from limitations in sensitivity that prevent their routine use for analyzing scarce samples. Some of these methods, such as microscopy and FACS are able to analyze the fluorescence of individual cells. However, nonspecific binding of cellular constituents other than the intended target protein(s) can lead to misleading signals. Towbin *et al.* (13) developed the Western blotting method to overcome this limitation of nonspecificity of antibodies, by electrophoretically separating proteins before antibody binding. However, conventional Western blotting typically requires relatively large numbers of cells, and is cumbersome and time consuming.

Capillary isoelectric focusing (IEF) has been shown to be powerful in its ability to resolve proteins (14, 15). Whole-capillary detection can significantly improve detection sensitivity over single-point detection methods (16, 17). Chemiluminescence may be used to improve detection sensitivity, employing off-column mixing of an analyte flume with chemiluminescence reagents (18). Immunoassays have been performed in capillary electrophoretic systems, involving formation of immune complexes which are then resolved in the capillary (19, 20). We have used aspects of each of these approaches, with the important addition of photochemical immobilization, to create a previously undescribed method that circumvents many of the limitations of existing methods for analyzing protein phosphorylation.

In this approach, proteins are resolved by IEF in a short length of capillary. Resolved proteins are then captured to the capillary wall by photochemically activated molecules lining the capillary. Such immobilization of the proteins allows immune complexes to be formed after the separation step, as a means of specific detection of target proteins. Because the protein–antibody complexes are immobilized in the capillary, chemiluminescence reagents can be flowed through the capillary, and light from the entire capillary can be imaged onto a CCD camera. Key to this approach is our utilization of the well-established idea of derivatizing the capillary wall with covalently bound acrylamide, for which multiple approaches exist (21–23). Similarly, we have used an established approach to photoimmobilization of proteins, with UV-activatable benzophenone (24, 25).

The key advantages of this approach are extraordinary sensitivity, and the ability to determine percent phosphorylation for each phosphorylation site of each isoform of a protein of interest. With respect to sensitivity, we demonstrate that we can resolve and detect isoforms of ERK protein from <25 cells. Also, using ERK as an example, we show that we can determine percent phosphorylation of ERK1 and ERK2 in a cell lysate sample. Singly and doubly phosphorylated forms of each isoform of ERK are resolved and their amount determined. Further, this

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Abbreviations: IEF, isoelectric focusing; PIF, probed isoelectric focusing.

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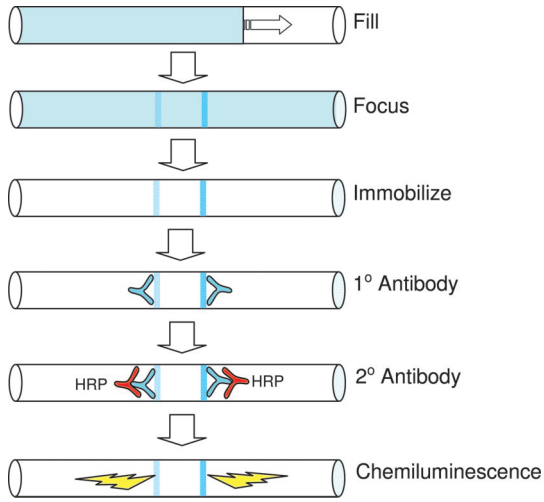

Fig. 1. PIF assay method. A 5 cm × 100 μm i.d. capillary is filled by touching its tip to a sample droplet. IEF is used to focus proteins and peptides in 4–30 min. Focused proteins are immobilized by 60-sec exposure to a UV light source. Primary antibody is flowed through the capillary, binding to its target protein. After washing, secondary antibody labeled with horseradish peroxidase (HRP) is flowed through the capillary, binding to the primary antibody. Finally, chemiluminescent reagents flowed through the capillary react with HRP to generate light, detected by a CCD camera.

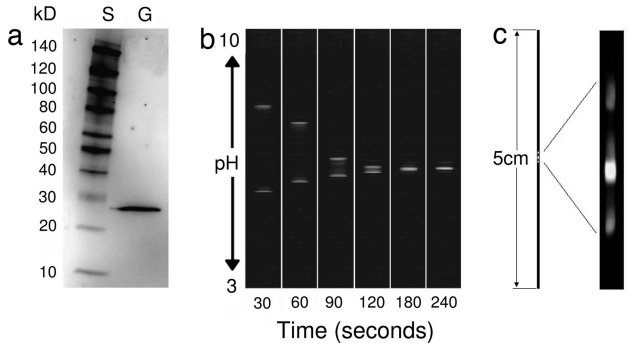

Fig. 2. Electrophoresis of GFP. (*a*) In a standard SDS/PAGE-based Western blot, only one protein band for GFP was revealed. (*b*) In a PIF capillary, GFP was resolved into one major and two minor forms in a pH 3–10 gradient. Focusing was complete in 4 min, visualized by fluorescence detection. (*c*) Closeup image of a section of the PIF capillary showing resolved forms.

quantitative analysis of ERK is achieved by analyzing the signal generated by using a single pan-specific antibody recognizing the ERK protein. For purposes of this discussion, we will abbreviate this previously undescribed method as probed isoelectric focusing (PIF).

Results

Assay Method. The PIF assay is performed in a 100 μm i.d. × 5 cm length of fused silica capillary with a clear coating to facilitate light transmission. The sample, typically a mixture of cell lysate and a separation buffer, initially fills the entire capillary. Fluorescent internal standards of known isoelectric points are also included in the mixture. Protein separation is achieved in 4 to 30 min by IEF. Immediately upon completion of separation, UV light is used to induce a photochemical reaction between photoactive moieties attached to the inner surface of the capillary, and proteins as well as fluorescent peptide standards resolved within the capillary. This results in covalent attachment of the analyte proteins and standards to the capillary, leaving an open central lumen through which reagents can be passed. After immobilization, wash solution, primary antibodies, and secondary antibodies are flowed through the capillary. The secondary antibody is typically labeled with the chemiluminescence reporter enzyme horseradish peroxidase (HRP). Finally, chemiluminescent reagents flowed through the capillary react with HRP to generate light, which is detected through the wall of the capillary. Excitation of the fluorescent standards and detection of the resulting fluorescent light allows the chemiluminescent and fluorescent signatures of the capillary to be superimposed. Typically twelve such capillary assays are run in parallel, and light is collected simultaneously from the full length of the 12 capillaries by using a CCD camera. Continuous streaming of the chemiluminescent reagents through the capillaries prevents their localized depletion, resulting in a linear response. The complete PIF process is illustrated (Fig. 1).

Apparatus. The apparatus required to perform a PIF assay is comprised of the following elements. A capillary holder maintains sample-filled capillaries in a horizontal position and exposes each of their ends to appropriate anolyte and catholyte solutions contained in wells. Electrodes immersed in these wells are used to apply a separation voltage. After focusing, a high-intensity UV light source is used to activate the immobilizing photochemistry. A source of vacuum is used to pull solutions through or out of the capillary. This is not required for filling the capillary, as a single capillary volume can be loaded into empty capillaries by capillary action. Vacuum is used to assist filling, to evacuate previously filled capillaries, and to pull multiple capillary volumes of wash buffer through the capillaries during wash steps. A CCD camera detects chemiluminescence and fluorescence signals. Light emitting diodes excite the fluorescent peptide internal standards.

Separation by IEF. The choice of isoelectric focusing for the analytical separation step is important for three reasons: (*i*) The separation is rapid. (*ii*) Multiple forms of a protein can be resolved, including isoforms and phosphorylated species. (*iii*) The entire capillary can be loaded with sample, contributing to high sensitivity. In contrast, size-based separations require narrow sample loading zones, severely limiting the amount of sample that may be loaded. Green fluorescent protein (GFP) analyzed by using a conventional SDS/PAGE Western blot revealed only a single band (Fig. 2*a*). In contrast, upon completion of IEF in a PIF capillary, GFP was resolved into three major forms (Fig. 2 *b* and *c*). These have been reported in the literature (26, 27). GFP was chosen in this example for its ability to be visualized by fluorescence during focusing. The purified recombinant GFP sample focused in 4 min. More complex samples such as cell lysates can take as long as 30 min to focus fully.

Quantitative Analysis of Phosphoforms and Isoforms. PIF can resolve the various phosphorylated forms and isoforms of signaling proteins. Often multiple capillaries are loaded with the same sample, and run through the PIF process. Each capillary is then probed with different antibodies, to determine which peaks correspond to specific protein forms. The reproducibility of the profile is high, and internal standards are used to align peaks. Thus, the profiles generated by using isoform-specific and phospho-specific antibodies can be compared with pan-specific antibody profiles. In this way, peaks within pan-specific antibody profiles may be identified as specific phosphoforms and isoforms of the target protein. Once individual peaks within the pan-specific antibody profile have been identified, integration of their signals allows determination of the relative quantities of isoforms and phosphoforms by using data from a single antibody. This avoids the problem of trying to quantify different protein forms with multiple antibodies having different binding avidities.

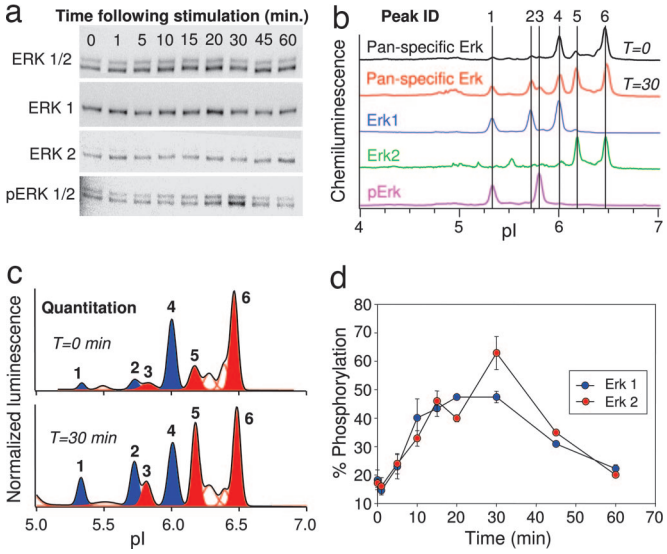

Fig. 3. Analysis of phosphorylated forms and isoforms of ERK protein. Cultured HT-29 human colorectal adenocarcinoma cells were treated with the cytokines insulin and TNF-α for 0 to 60 min before preparation of lysates. (*a*) Conventional SDS/PAGE Western blot of 0- to 60-min samples, probed with antibodies specific for ERK1, ERK2, ERK1/2 (pan-specific ERK antibody), and phosphorylated ERK1&2 (pERK1/2). (*b*) PIF analysis of these samples, probed with the same antibodies allowed peak identification as follows: 1, ppERK1; 2, pERK1; 3, ppERK2; 4, ERK1; 5, pERK2; and 6, ERK2. (*c*) Differences in phosphorylation upon stimulation are evident in the pan-specific Ab profiles obtained for 0- and 30-min lysates. Integration of the peaks corresponding to each identified species allows percent phosphorylation of ERK1 and ERK2 to be calculated. (*d*) Using percent phosphorylation data generated as in *c* allowed phosphorylation vs. time of cytokine stimulation to be plotted for both ERK1 and ERK2. Percent phosphorylation shown is the sum of peak integrations for mono- and diphosphorylated species.

Quantitative Analysis of ERK Protein. Multiple isoforms and phosphorylated forms of endogenous ERK, a key cell signaling protein, were studied in HT-29 human colon adenocarcinoma cultured cells. ERK1 and ERK2 are also known as p44 and 42 MAP kinase, respectively, and also as MAPK1 and MAPK2, respectively. Dual phosphorylations at Thr-202/Tyr-204 for ERK1, and the corresponding positions Thr-185/Tyr-187 for ERK2, are known to activate these closely related proteins (28). Phosphorylation of either of these residues can occur independently of the other (29). Mechanistic studies of ERK phosphorylation have indicated that phosphorylation is distributive rather than processive; each phosphorylation occurs by independent enzyme–substrate interactions, rather than successively during a single enzyme–substrate interaction (30).

Cell lysates were generated from HT-29 human colon adenocarcinoma cells treated with insulin plus TNF-α for 0 min (no treatment) to 60 min. Analysis of these lysates by conventional SDS/PAGE Western blots (Fig. 3*a*) was performed by using pan-specific (ERK 1/2), isoform-specific (ERK1 and ERK2) and phosphorylation-specific (pERK1/2) antibodies. Although differences in ERK phosphorylation were evident from probings with the phosphorylation-specific antibody, it was not possible to determine percent phosphorylation by using the conventional Western blot, as different antibodies typically exhibit different binding avidities for their respective epitope targets. PIF analysis of these same samples (Fig. 3 *b–d*) revealed differences upon stimulation in the phosphorylation of ERK1 and ERK2. Capillaries probed with isoform- and phosphoform-specific antibodies allowed six significant peaks to be identified as the nonphosphorylated and phosphorylated forms of ERK1 and ERK2 (Fig. 3*b*). All six of these forms were identifiable in the profile generated by using pan-specific antibody recognizing both isoforms. Peak 1, identified by pan-specific-, ERK1- and phospho-ERK-specific antibodies, and the most acidic ERK form identified, is consistent with an identity of diphospho-ERK1. Peak 2, identified by pan-specific- and ERK1-specific antibodies, but not by phospho-ERK-specific antibody, and the second most acidic ERK form identified, is consistent with an identity as monophospho-ERK1, assuming the phospho-specific antibody used does not recognize monophosphorylated forms of ERK. According to the manufacturer, the phospho-specific monoclonal antibody used was generated against a diphosphorylated peptide. Peak 3 was the most acidic of the peaks recognized by the ERK2-specific antibody, and was also recognized by the phospho-specific antibody, consistent with its identity as diphospho-ERK2. Peak 4, identified by pan-specific and ERK1-specific antibodies, but not by the phospho-specific antibody, was the most basic ERK1-antibody-positive peak, consistent with its identity as nonphosphorylated ERK1. Peak 5 was recognized by the pan-specific- and ERK2-specific antibodies, but not by the phospho-specific antibody. As was the case for peak 2, assuming that the antibody used does not recognize mono-phosphorylated ERK, peak five's position in the isoelectrophoretic separation, and its recognition by specific antibodies, is consistent with its identity as monophosphorylated ERK2. Peak 6, the most basic peak recognized by the pan-specific antibody, also recognized by ERK2 specific antibody, is consistent with an identity as nonphosphorylated ERK2.

In addition to the above-identified species, three peaks with apparent pI values of 5.50, 6.29, and 6.40 were recognized by the pan-specific antibody used, but not by the ERK1- or phospho-specific-antibodies. Some level of binding to these peaks by the ERK2-specific antibody may be evident. It was not possible to assign identity to these peaks with any certainty, as they did not correspond to any of the species expected to be present. Treatment of samples with exogenously added phosphatase eliminated all peaks identified above as being due to phosphorylated species (data not shown). The unknowns described above, as well as the peaks identified as due to nonphosphorylated ERK1 and ERK2 were retained upon phosphatase treatment.

Having thus separated and identified the expected ERK species present, the relative amounts of these could be determined by integration of the pan-specific antibody profiles. Changes in these profiles upon cytokine stimulation of cells used to prepare lysates were evident. Profiles at 0 min and 30 min postcytokine-stimulation are shown with individual peak integration (Fig. 3*c*). A detailed profile of ERK1 and ERK2 phosphorylation as a function of time of cytokine stimulation is also shown (Fig. 3*d*). This analysis revealed that before cytokine stimulation, nonphosphorylated ERK1 and ERK2 represented in excess of 80% of the ERK species present. The peak of phosphorylation occurred at 30 min, at which time $\approx$82% of ERK2 was either singly or doubly phosphorylated, and $\approx$47% of ERK1 was either singly or doubly phosphorylated (Fig. 3*d* and Table 1). Similar results to these, but generated by using a conventional Western blot approach have been reported (31). The Western blots used in that study allowed determination of relative phosphorylation of ERK, but not percent phosphorylation, or the relative amounts of ERK 1 vs. ERK 2 phosphorylation, or of monophosphorylated vs. diphosphorylated species.

Isoelectric Point Determination. Each PIF capillary was loaded with fluorescence-labeled synthetic peptides as internal isoelectric-point standards. These were detected separately from the chemiluminescence signal by means of LED-excited fluorescence, using the CCD camera also used for chemiluminescence. These standards, although similar in general concept to those of Shimura *et al.* (32), differ in their use of D-amino acids to prevent proteolytic degradation by lysates. Determination of the isoelec-

Table 1. Summary of peak data for endogenous ERK from cytokine-stimulated HT-29 cells

No.	Assigned identity	Determined isoelectric point	Calculated isoelectric point*	Area %† $T = 0$	SD,† %	Area %† $T = 30$	SD,† %
1	Diphospho-ERK1	5.33	6.01	9	3	20	2
	Unknown	5.50					
2	Monophospho-ERK1	5.72	6.1	9	4	27	2
3	Diphospho-ERK2	5.81	6.15	6	4	8	0
4	ERK1	6.01	6.28	82	4	53	2
5	Monophospho-ERK2	6.19	6.38	12	4	55	6
	Unknown	6.29					
	Unknown	6.40					
6	ERK2	6.48	6.53	83	2	37	6

*From Scansite.
†Mean and SD from three replicate Firefly assays.

tric points of these standards, and their use in calibrating PIF capillaries, is described in *Methods*.

Generalizability of PIF Method. The generalizability of the PIF method was demonstrated by testing its ability to produce a reproducible pattern for a variety of specific proteins in cell lysates, using their respective pan-specific antibodies for detection. Endogenous Akt was detected in lysates of LNCaP cultured prostate cancer cells, and produced consistent profiles by using a pan-specific antibody for detection (Fig. 4*a*). Further, specific peaks phosphorylated at S473 were readily identified by using a commercially available pS473-specific antibody (data not shown). The isoelectric points indicated by PIF for peaks identified as Akt by using the pan-specific antibody (pI range 5.95–5.35) were consistent with isoelectric points calculated for Akt isoforms 1, 2, and 3 containing zero to four phosphorylations (pI range 5.75–5.48) calculated by using Scansite (33). Akt has been reported to contain four phosphorylatable amino acid residues (34). β-Catenin was also identified in lysates of LNCaP cells by using a pan-specific β-catenin antibody (data not shown). The pattern for β-catenin was consistent between capillaries, and

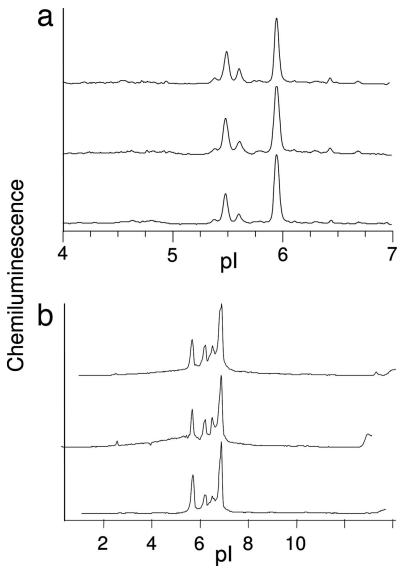

Fig. 4. Generalizability of the PIF method for analyzing signaling proteins in cell lysates. For each protein target, profiles from three separate capillaries are shown. (*a*) Endogenous Akt protein in LNCaP cell lysate probed with a pan-specific Akt antibody. (*b*) Recombinant human BCL2 protein spiked into LNCaP cell lysate, probed with a pan-specific BCL2 antibody.

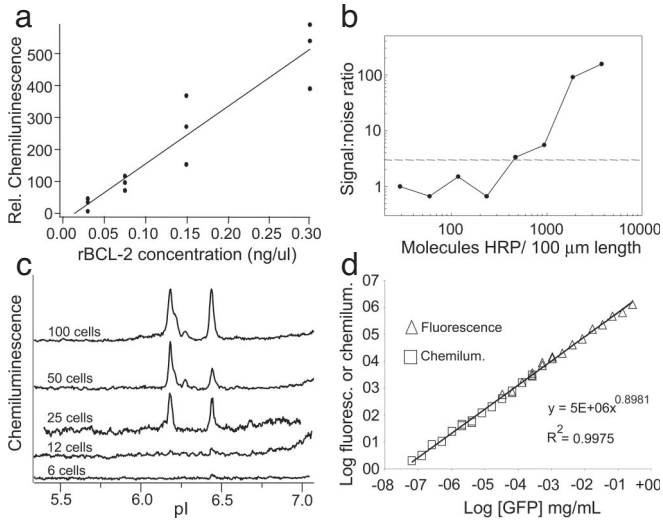

Fig. 5. Sensitivity, limit of detection, and dynamic range of capture for PIF assay. (*a*) The limit of detection for recombinant BCL2 added at known concentrations to LNCaP cell lysates was 2 pg/μl. (*b*) The limit of detection for HRP enzyme plus luminol/peroxide substrate imaged by the CCD camera in a capillary shows <500 molecules of HRP per 100-μm segment of capillary (corresponding to the width of a focused peak) gave a 3:1 signal:noise ratio. (*c*) Detection of endogenous ERK protein in lysate of LNCaP cells. The amount of lysate in a capillary corresponds to the number of cells indicated. (*d*) Log-log plot of GFP protein capture shows linearity of capture over >6 orders of magnitude in protein concentration. Detection was by GFP fluorescence and HRP-Ab-based chemiluminescence.

the indicated isoelectric point for the major peak was 5.9, relatively consistent with the isoelectric point for the nonphosphorylated form of the human protein of 5.53, calculated by using Scansite. Recombinant human BCL2 was spiked into LNCaP cell lysate and detected with a pan-specific antibody (Fig. 4*b*). Four significant peaks were detected, consistent with one of these being nonphosphorylated protein, and the other three having one, two, and three of the three known phosphorylation sites (35) occupied. The indicated isoelectric points by PIF assay ranged from 6.9–5.75, relatively consistent with isoelectric points of BCL2 α and β forms containing 0 to 3 phosphorylations (pI from 6.75–5.84) calculated by using Scansite.

Sensitivity and Limit of Detection. The sensitivity of the system was determined by spiking known amounts of recombinant BCL2 protein into cell lysates not otherwise producing BCL2-antibody-positive peaks at the locations where this protein was resolved. The concentration at which a 3:1 signal:noise ratio was achieved was 2 pg/μl. A response vs. protein concentration plot is shown for assays performed in triplicate (Fig. 5*a*). Details of the statistical method used to determine limit of detection are given in *Methods*.

Another approach to determining the detection limit, specifically for the chemiluminescent detection system used for PIF, was to fill capillaries with known concentrations of HRP, the detection enzyme used in PIF assays. In this case, the solution filling the capillaries also contained our standard concentration of chemiluminescent reagent (luminol plus peroxide). Light was collected by using the CCD camera. By examining the signal emitted by a 100-μm-long section of the capillary (corresponding to a typical peak width of a resolved protein) the amount of HRP that generated a 3:1 signal:noise ratio was determined to be <500 molecules (Fig. 5*b*).

The signal from endogenous ERK in LNCaP cell lysates made from varying numbers of cells was also measured (Fig. 5*c*). For

this work, cells were released from plates by trypsin treatment, counted, and diluted with fresh buffer before lysis. The number of cells represented by the material actually delivered in the 400 μl volume of the capillaries is indicated. Given some expected loss of cells in this preparation process, the number given is an upper limit of the number of cells represented by the contents of the capillary. It can be seen that 25 cells worth of lysate delivered to the capillary provided detectable ERK signal with the recognizable ERK1/ERK2 profile seen at higher sample concentrations.

The dependency between protein concentration and capture efficiency was examined by using GFP protein at varying concentrations in HNTG buffer plus all focusing buffer components loaded into capillaries. After loading and focusing in a pH 3–10 gradient, protein was immobilized by UV activation. GFP fluorescence measurements were then taken before and after flushing unbound GFP out of the capillary. In a parallel set of assays, chemiluminescence was used for detection, using an HRP-labeled antibody recognizing GFP. Two important points were determined by these experiments: (*i*) binding as a function of protein concentration was linear over more than six orders of magnitude (Fig. 5*d*), and (*ii*) the proportion of GFP protein immobilized was $\approx$0.01% (data not shown).

Discussion

Sanger sequencing and Western blotting are two of the most commonly used methods in biology. Sanger (36) published his method for sequencing DNA by using dideoxy nucleotide terminators in 1977, just 2 years before the introduction of Western blotting. Since that time, a series of technological advancements revolutionized how Sanger sequencing is used to analyze DNA (37). This enabled the first draft sequencing of the human genome in 2000. Remarkably, in this same period, Western blotting has remained little changed.

Western blotting, although powerful and widely used, suffers several limitations. Among these are the time and number of manual steps required, the relatively large amount of material required, and difficulty in quantitatively determining protein phosphorylation. The PIF assay in some respects parallels the Western blotting method, while overcoming many of its limitations, as follows. The resolution of capillary IEF combined with rapid in-capillary immobilization allows protein isoforms and phosphoforms to be resolved and quantified by using a single pan-specific antibody. The small volume of the capillary combined with the use of flowed chemiluminescence substrates and whole-capillary imaging contribute to high-sensitivity. Although not discussed in detail here, the capillary format also lends itself to automation, as it is easily handled by conventional robotics approaches, as the assay remains in the same capillary throughout the entire process.

In practice, we believe the PIF assay is likely to find particular utility in the analysis of scarce samples such as fine needle aspirates from small animals or patients, and scarce FACS-sorted cells such as stem cells. In one mode of assay, the small sample consumption allows many capillaries to be filled from a single sample consisting of only a few microliters, allowing different antibodies to be used to probe many capillaries run in parallel. Single cell capillary electrophoresis has been previously demonstrated by using fluorescence detection (38). Given this, our current cell limit of detection, and the small fraction of available protein captured in the current assay, one may envision as the method evolves performing PIF on the contents of a single cell.

Materials and Methods

Cell Culture. The HT-29 human colorectal adenocarcinoma cell line was from ATCC (Manassas, VA; HTB-38), and grown in McCoy's 5a medium. For cytokine stimulation experiments, these cells were treated with 500 ng/ml insulin plus 100 ng/ml TNF-α. The LNCaP human prostate cancer cell line was from ATCC (CRL-1740), and grown in RPMI medium 1640. See *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site, for details.

Lysate Preparation. Cells were lysed in HNTG buffer (20 mM Hepes, pH 7.5/25 mM NaCl/0.1% Triton X-100/10% glycerol, freshly prepared with 0.1% protease inhibitor mixture (539134; Calbiochem, San Diego, CA) and 1% phosphatase inhibitor mixture (p2850; Sigma, St. Louis, MO), and clarified by centrifugation. Protein concentration was determined by using a bicinchoninate assay (23225; Pierce, Rockford, IL). Some samples were enzymatically dephosphorylated by combining 8 μl cell lysate with 1 μl 40 mM $MnCl_2$ and 125 units lambda phosphatase (14-405; Upstate Biotechnology, Charlottesville, VA), incubating for 1 h at 37°C. See *Supporting Materials and Methods* for details.

Sample Preparation for IEF. Cell lysate in HNTG buffer was combined with an equal volume of IEF buffer solution consisting of 20% sorbitol/0.1 M NDSB-256 (D465250; Toronto Research Chemicals, North York, ON, Canada)/2 mM of each fluorescent peptide standard/10% vol/vol ampholyte solution. Broad range ampholytes, pI 3–10, were from Sigma (1522). Narrow range ampholytes were from Bioworld (Dublin, Ohio), as follows: pI 4–7 (764050); pI 5–8 (764058), and; pI 5–7 (764056). GFP protein was prepared at a final concentration of 0.02 mg/ml in HNTG buffer and combined 1:1 with IEF solution as described above.

Capillary Preparation. A 100-μm i.d. x 375-μm o.d. Teflon-coated fused silica capillary with interior vinyl coating (TSU 100375; Polymicro Technologies, Phoenix, AZ) was surface grafted with polyacrylamide copolymerized with 1 mol percent acrylbenzophenone. Identically prepared capillary is available from Cell Biosciences (400100).

Apparatus. Prototype systems using the following essential components were used. The electrophoresis power supply was a model J4–3P from Matsusada Precision (San Jose, CA). The UV light was a 1300M lamp from Fusion UV Systems (Gaithersburg, MD) at 8 cm distance from capillaries. The CCD camera was a PIXIS model 1024B from Princeton Instruments (Trenton, NJ). Light collection was through a model DGRF 20 mm f1.8 lens from Sigma (Kanagawa, Japan). Excitation of peptide standards and GFP was by 16 Green (532 nm) LEDs, from LITE-ON Technology (Milpitas, CA; LTL2T3TGK6), with a combined output of 40 candelas. Excitation (HQ525/50X) and emission (HQ600/50M) filters were from Chroma Technology (Rockingham, VT).

IEF and Immobilization. Samples (5–10 μl) prepared as above were contacted with the tip of a capillary and mild vacuum was applied to facilitate loading. Capillaries were then placed horizontally in contact with buffer reservoirs containing 10 mM H_3PO_4 (anodic end) and 400 mM NaOH (cathodic end). Generally, an initial potential of 100 V was applied for 700 s, followed by 200 V for 700 s, then 1500 V for 1000 s. GFP was focused at 900 V potential applied for 240 s. Proteins were immobilized by 15–60 s irradiation with UV light.

Washing and Probing. After immobilization capillaries were washed with a TBST solution consisting of 10 mM Tris·HCl/150 mM NaCl/0.05% Tween 20, pH 6.8. A 5 mm Hg vacuum source was applied to the cathodic end to pull TBST solution through each capillary for 5 min. Similarly, primary antibody solutions at dilutions ranging from 1:100 to 1:10,000 were drawn into capillaries by a 5-s application of vacuum. Antibody incubations were 10 min. This

antibody application procedure was repeated a total of five times. After a 5-min TBST wash, secondary antibodies were similarly applied, but with 5-min instead of 10-min incubations.

Antibodies and Recombinant Proteins. GFP antibody was from Invitrogen (Carlsbad, CA; A11122). Recombinant GFP was from Clontech (Mountain View, CA; 632373). ERK pan-specific antibody was from Upstate (06-182). ERK 1 antibody was from Upstate (05-957). ERK 2 antibody was from Cell Signaling Technology (Danvers, MA; CS9197). Phospho-ERK antibody was from Upstate (05-797). Akt pan-specific antibody was from Cell Signaling Technology (CS 9272). Akt pS473 antibody was from Cell Signaling Technology (CS 4058). β-Catenin pan-specific antibody was from Santa Cruz Biotechnology (Santa Cruz, CA; SC 7199). Recombinant BCL2 was from R & D Systems (Minneapolis, MN; 827-BC). BCL2 antibody was from Becton-Dickinson (Franklin Lakes, NJ; BD 51–1513GR). Secondary antibody used was a 1:10,000 dilution of anti-rabbit-HRP in TBST (81–6120; Zymed, South San Francisco, CA). SDS/PAGE standards and antibody for detecting them on Western blots were from Cell Signaling Technology (7727).

Fluorescent Peptide Standards. Tetramethylrhodamine-labeled fluorescent peptide standards were made by using Fmoc chemistry and D-amino acids on an ABI 433A peptide synthesizer. Their isoelectric points were determined by measuring the pI of the gel in the region of each focused band in a conventional IEF gel as described (32). These standards are available from Cell Biosciences.

Chemiluminescence Detection. A mixture of equal parts SuperSignal West Femto Stable Peroxide buffer and Luminol/Enhancer solution from Pierce (1859023 and 1859022, respectively) was pulled through the capillaries with a 5-mm Hg vacuum. Capillaries were laid horizontally in a capillary holder, and a slight excess of chemiluminescent reagent mix was supplied to one of the two reservoirs to create a hydrostatic head and resulting continuous flow of reagent. Chemiluminescence signal was collected for 60 s to 3 min depending on signal strength.

Data and Statistical Analysis. Chemiluminescece and fluorescence data were extracted from CCD images of capillaries by drawing a straight line down the center of the capillary and extracting a signal profile. Peaks corresponding to fluorescent standards were identified and the positions of peak centers were tabulated. A linear regression fit was used to generate a function relating pI value to pixel position. This function was used to calculate the pI value of each pixel in the raw chemiluminescence isoelectropherogram, converting signal vs. pixel data into signal vs. pH data. Multipeak fitting and peak area calculations were done with Peak Fit v4.11 (Systat Software, Point Richmond, CA), using Gaussian peaks with variable widths. Further details are given in *Supporting Materials and Methods.*

rBCL2 Limit of Detection. The best fit line was calculated by linear regression with IGOR Pro version 5.03 (WaveMetrics, Lake Oswego, OR). Uncertainties in slope and intercept were used as reported by IGOR Pro but can be calculated by methods described elsewhere (39). Standard deviation of the baseline adjacent to the measured peak from each data set was calculated with IGOR Pro. The limit of detection, defined as 3 times the standard deviation of the baseline divided by the slope of the regression line is reported. Further details are given in *Supporting Materials and Methods.*

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1. Blume-Jensen P, Hunter T (2001) *Nature* 411:355–365.
2. Ullrich A, Schlessinger J (1990) *Cell* 61:203–212.
3. Gennaro G, Ménard C, Giasson E, Michaud S-É, Palasis M, Meloche A, Rivard A (2006) *Arterioscler Thromb Vasc Biol* 23:204–210.
4. Zhang Q, Nottke A, Goodman RH (2005) *Proc Natl Acad Sci USA* 102:2802–2807.
5. Sadick MD, Sliwkowski MX, Nuijens A, Bald L, Chiang N, Lofgren JA, Wong WL (1996) *Anal Biochem* 235:207–214.
6. Ahn JS, Musacchio A, Mapelli M, Ni J, Scinto L, Stein R, Kosok K, Yeh L-I (2004) *J Biomol Screen* 9:122–131.
7. Rijksen G, Van Oirschot BBA, Staal GE (1989) *Anal Biochem* 182:98–102.
8. Gembitsky DS, Lawlor K, Jacovina A, Yaneva M, Tempst P (2004) *Mol Cell Proteomics* 3:1102–1118.
9. Sachs K, Perez O, Pe'er D, Lauffenburger DA, Nolan GP (2005) *Science* 308:523–529.
10. Preuss U, Mandelkow E-M (1998) *Eur J Cell Biol* 76:176–184.
11. Gerber SA, Rush J, Stemman O, Kirshner MW, Gygi SP (2003) *Proc Natl Acad Sci USA* 12:6940–6945.
12. Zhou H, Watts JD, Aebersold R (2001) *Nat Biotechnol* 4:317–318.
13. Towbin H, Staehlin T, Gordon J (1979) *Proc Natl Acad Sci USA* 76:4350–4354.
14. Graf M Watzig H (2004) *Electrophoresis* 25:2959–2964.
15. Righetti PG, Gelfi C, Conti M (1979) *J Chromatogr B* 699:91–104.
16. Wu X-Z, Huang T, Mullett WM, Yeung JM, Pawliszyn J (2001) *J Microcolumn Sep* 13:322–326.
17. Wu X-Z, Pawliszyn J (2002) *Electrophoresis* 23:542–549.
18. Tsukagoshi K, Nakahama K Nakajima R (2004) *Anal Sci* 20:379–381.
19. Shimura K, Karger B (1994) *Anal Chem* 66:9–15.
20. Cunliffe JM, Liu Z, Pawliszyn J, Kennedy RT (2004) *Electrophoresis* 25:2319–2325.
21. Cobb KA, Dolnik V Novotny M (1990) *Anal Chem* 62:2478–2483.
22. Gelfi C, Curcio M, Righetti PG, Sebastiano R, Citterio A, Ahmadzadeh H, Dovichi NJ (1998) *Electrophoresis* 19:1677–1682.
23. Gao L, Liu S (2004) *Anal Chem* 76:7179–7186.
24. Dormán G, Prestwich GD (2000) *Trends Biotechnol* 18:64–77.
25. Janecki DJ, Broshears WC, Reilly JP (2004) *Anal Chem* 76:6643–6650.
26. Korf GM, Landers JP, O'Kane DJ (1997) *Anal Biochem* 251:210–218.
27. Richards DP, Stathhakis C, Polakowski R, Ahmadzadeh H Dovichi NJ (1999) *J Chromatogr A* 853:21–25.
28. Seger R, Ahn NG, Boulton TG, Yancapoulos GD, Panayotatos N, Radziejewska E, Ericsson L, Bratlien RL, Cobb MH, Krebs EG *Proc Natl Acad Sci USA* 88:6142–6146.
29. Her J-H, Lakhani S, Zu K, Vila J, Dent P, Sturgill TW, Weber MJ (1993) *Biochem J* 296:25–31.
30. Ferrell JE, Bhatt RR (1997) *J Biol Chem* 272:19008–19016.
31. Gaudet S, Janes KA, Albeck JG, Pace EA, Lauffenburger DA, Sorger PK (2005) *Mol Cell Proteomics* 4:1569–1590.
32. Shimura K, Kamiya K, Matsumoto H, Kasai K (2002) *Anal Chem* 74:1046–1053.
33. Obenauer JC, Cantley LC, Yaffe MB (2003) *Nucleic Acids Res* 13:3635–3641.
34. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BH (1996) *EMBO J* 23:6541–6551.
35. Yamamoto K, Ichijo H, Korsmeyer SJ (1999) *Mol Cell Biol* 19:8469–8478.
36. Sanger F, Nicklen S Coulson AR (1977) *Proc Natl Acad Sci USA* 74:5463–5467.
37. Dovichi NJ, Zhang J (2000) *Angew Chem Int Ed* 39:4463–4468.
38. Hu S, Zhang L, Krylov S Dovichi NJ (2003) *Anal Chem* 75:3495–3501.
39. Roser B (1990) *Fundamentals of Biostatistics* (PWS-KENT, Boston, MA), pp 398–473.