We developed a differential method to reveal kinase-specific phosphorylation events in live cells. In this method, cells in which the specified kinase is inactive are labeled with $^{32}\text{Pi}$, whereas cells in which the kinase is active are labeled with $^{33}\text{Pi}$. The two cell extracts are then mixed, and proteins are separated on a single two-dimensional gel. The dried gel is exposed twice. The first exposure reveals both $^{32}\text{P}$- and $^{33}\text{P}$-labeled proteins; the kinase-specific spots are revealed because of $^{33}\text{P}$ labeling. The second exposure is conducted with two acetate sheets intervening between the gel and the detection plate. This maneuver screens out the less energetic $^{33}\text{P}$-labeled proteins while allowing the more energetic $^{32}\text{P}$-labeled proteins to be detected, thus leaving only those spots that were phosphorylated independently of the specified kinase. We demonstrate the utility of this method for detecting kinase substrates in rare tissue by focusing on extracellular signal-regulated kinase-specific phosphorylation of stathmin/OP18 in primary rat sympathetic neurons.

Phosphorylation is a major reversible protein modification. It regulates a myriad of protein functions including enzyme activity, protein-protein interaction, cellular localization, and protein degradation. It is estimated that there are about 100,000 potential phosphorylation sites in the human proteome of which fewer than 2000 are currently known (1). In addition to identification of phosphorylation sites, there is a need for quantitation of phosphorylation events especially with regard to understanding the regulation of signal transduction. Although novel and sophisticated methods have been developed to enrich for phosphoproteins or phosphopeptides prior to analysis by mass spectrometry, this approach requires large amounts of protein and so is not always feasible when using rare tissue. Moreover from the standpoint of systems biology, it may be useful to acquire an image of protein phosphorylation of the entire proteome prior to homing in on specific proteins.

Labeling cells with phosphorus-32 ($^{32}\text{P}$) has long been used as a means for identifying phosphoproteins. Principle $\beta$ emission energy for $^{32}\text{P}$ is 1.709 MeV, making it highly sensitive, and its rapid uptake into cellular ATP makes it very versatile. Although it is possible to run simultaneous gels to compare changes in phosphoprotein profiles between differentially treated samples, it would be an advantage to be able to discriminate changes in phosphorylation between two samples on a single 2D gel just as DIGE is used to highlight changes in protein expression between two samples while eliminating the variability of protein separation patterns (2). We previously noted that it is possible to prelabel cellular proteins metabolically with $[^{35}\text{S}]$methionine (principle $\beta$ emission, 0.167 MeV) and then label the same cells with $^{32}\text{P}$ to detect which of these proteins are phosphorylated; as $^{35}\text{S}$ emission has lower energy it is possible to screen out the lower energy using a simple device such as an acetate sheet while still permitting $^{35}\text{S}$ radiation to be detected (3). The maximum $\beta$ emission energy for phosphorus-33 ($^{33}\text{P}$) is 0.249 MeV, which is about 6.8 times lower than that of $^{32}\text{P}$. Hence we reasoned that it might be possible to use a similar configuration by mixing differentially treated samples, one labeled with $^{33}\text{P}$ and the other labeled with $^{33}\text{P}$, and running them on a single 2D gel. This would maximize yield (especially when dealing with small samples such as rare tissue, e.g. primary neurons) while eliminating ambiguity in spot detection due to differences between the patterns of two 2D gels. Here we demonstrate the utility of this method by focusing on phosphostathmin as our test protein and primary rat superior cervical ganglion (SCG) neurons as our cell type. We chose this system because it is a good example of a rare tissue type; each ganglion yields only about 10,000 highly purified neurons.

1 The abbreviations used are; 2D, two-dimensional; ERK, extracellular signal-regulated kinase; DIPPL, differential phosphoprotein labeling; araC, cytosine arabinoside; CPTcAMP, 8-(4-chlorophenylthio)-cAMP; NGF, nerve growth factor; SCG, superior cervical ganglion; PKA, cAMP-dependent protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

2 B. Amess, unpublished data.
Stathmin is a 19-kDa protein that integrates via its phosphorylation several different signaling pathways (4–8). At least 16 molecular forms of stathmin have been identified that migrate as two unphosphorylated forms (α and β; the α form is usually more abundant) and seven increasingly phosphorylated spots each with molecular mass of 19–23 kDa and pI of 6.2 to >5.6. Strikingly there are typically three sizes or “tiers” of migration of these phosphoproteins on SDS-PAGE. “Set 1” consists of two unphosphorylated forms, that migrate at about 19 kDa with pI values of >6, 5.8, and 5.6, respectively, due to addition of one, two, or three phosphates (labeled P1, P2, and P3). Tier 2, “set 16,” is comprised of α1 and α2 that migrate at about 21 kDa with pI values of about 5.8 (P2) and 5.6 (P3). Tier 3, “set 17,” is comprised of α2 and α3 that migrate at about 23 kDa with pI values of 5.6 (P3) and >5.5 (P4). The β form is modified similarly except that it is slightly more acidic. In vitro, PKA and Cdc2 phosphorylate stathmin on two mutually exclusive serines each, giving rise to the complete pattern described above (4). In PC12 cells, phosphorylation of set 16 and set 17 is induced by NGF among which serine 25 (the site phosphorylated by Cdc2 in vitro) is also heavily induced by mitogen-activated protein kinase/ERK (9). Serine 38 is moderately phosphorylated as well. We have shown previously that NGF maintains prolonged ERK activity in SCG neurons (10, 11) without activating PKA (12). Moreover SCG neurons do not express Cdc2 (13). Knowing the pattern of migration of stathmin and using the cyclic AMP analogue 8-(4-chlorophenylthio)-cAMP (CPTcAMP) were from Sigma, and phosphate-free RPMI1640 medium was from ICN Biomedicals (Eastman Kodak Co.). CPTcAMP was used as a kinase/ERK inhibitor, 100 ng/ml NGF (added from a 200 µg/ml concentrate to retain steady state labeling) was added to both experimental and control dishes for an additional 3 h. For exploratory work, SCG neurons were labeled with 0.5 mcCi of 32P or 33P for 3.5 h but lysed immediately in one-dimensional Laemmli sample buffer (16).

**Sample Preparation**—Neurons were carefully washed three to four times in L15-CO2 medium (containing phosphate) without serum, scrapped off in 0.5 ml of medium containing 0.01% BSA, and left on ice for 5 min after which neurons were pelleted by spinning for 3 min at 4000 rpm in a microcentrifuge. The pellet containing the neurons was washed with ice-cold PBS or medium without any additions and lysed in 320 µl of IPG buffer containing 7 µl urea, 2 µl thiourea, 4% CHAPS (or alternatively 2% ASB-14), 1.2% Pharmalytes pH 3–10, 20 mM DTT, 10 mM Tris-HCl, pH 8, and bromphenol blue. The pellet was vortexed rigorously a few times for 30–60 s each time until complete solubilization was achieved and centrifuged at maximum speed in a microcentrifuge to remove any membrane material. Approximately 1% of the volume was subjected to TCA precipitation and scintillation counting to estimate the total amount of 32P and 33P incorporation and thus adjust the respective total label content between experimental and control samples before mixing both samples (see text for additional comments). TCA precipitation was conducted in 25% ice-cold TCA (1 ml) followed by decantation onto a Whatman GF/C glass fiber filter and four washes with ice-cold 5% TCA as described previously (3).

**Gel Electrophoresis**—For the first dimension of the 2D separation, each sample was absorbed into an inverted immobilized pH gradient gel (Immobiline DryStrip, pH 3–10 nonlinear, 18 cm, Amersham Biosciences) during an overnight incubation at room temperature in a reswelling tray to allow uptake of the proteins. Isoelectric focusing was performed with a mineral oil overlay in a Multiphor II flat bed electrophoresis unit (Amersham Biosciences) set at 2 mA, 5 watts and ramped to 100 V for 1 h, 300 V for 1 h, 500 V for 1 h, 3500 V for 3.5 h, and finally 3500 V for 12 h. The strips were then equilibrated for 10–15 min with gentle shaking in a buffer containing 50 mM Tris-HCl, pH 6.8, 2% SDS, 6 µl urea, 30% glycerol, and 20 mM DTT and placed on top of the second dimension gel. Proteins were separated by SDS-PAGE on a 20 × 20-cm gel containing 11% acrylamide using an in-house built apparatus (3). Protein standards (2D Bio-Rad markers) were run along with the samples to ensure equivalent patterns of protein separation between gels. For exploratory work, proteins were separated by SDS-PAGE on an 8 × 10-cm minigel, stained, dried, and imaged as above. 14C-Rainbow markers (catalog number CFA756) were from Amersham Biosciences.

**Image Analysis**—Gels were stained (and fixed) in Coomassie Blue solution, destained, dried, and exposed to a phosphorimaging screen (Eastman Kodak Co.). Screens were scanned at 88-µm resolution using an Amersham Biosciences PhosphorImager 425 as described previously (3). Data was stored as a 16-bit tiff. In some cases, acetic sheets (PPCI-SC/LE OHP photocopier film, Lloyd Paton Ltd., Manchester, UK) were placed between the screen and the dried gel as described below. The Coomassie-stained gel image was obtained by scanning with a Hewlett Packard Scanjet 5470C flat bed scanner. Raw images were imported into NIH Image 1.62 to quantify intensity of bands/spots.
RESULTS AND DISCUSSION

We first examined whether it is feasible to use acetate sheets to screen out $^{33}$P signals without affecting $^{32}$P detection. Extracts from $^{33}$P- or $^{32}$P-labeled neurons (each labeled with 0.5 mCi of respective radionuclide) were separated either on two separate lanes of a one-dimensional gel (20 μl of each extract) or mixed and separated as a single sample (e.g. 40 μl of the mixture loaded). Fig. 1A, left, shows results obtained after exposure of the dried gel to a phosphorimaging screen for 20 h, while on the right is the same gel re-exposed for 30 h with two acetate sheets interposed between the gel and the screen. The intensity of the bands marked $i$, $ii$, and $iii$ is given in Table I.

![Image of gel electrophoresis results](image)

FIG. 1. Testing the principle of using acetate sheets to block selectively $^{33}$P emission in a mixed sample of $^{33}$P/$^{32}$P-labeled proteins. A, SCG neurons (about 200,000 neurons/sample) were labeled with 0.5 mCi of $^{32}$P or $^{33}$P for 3.5 h and lysed in 40 μl of SDS-PAGE sample buffer. A 20-μl aliquot of $^{33}$P-labeled (lane 1) or $^{32}$P-labeled (lane 2) proteins was separated on a 10% SDS gel alongside a mixed sample containing 20 μl of each of the $^{33}$P- and $^{32}$P-labeled proteins (lane 3). $^{14}$C-Labeled molecular weight markers were run in lane 1. Gels were dried and exposed to a phosphorimaging screen once without (left, 20 h) and then with (right, 30 h) two acetate sheets intervening between the gel and the screen. Note the loss of $^{33}$P labeling in lanes 1 and 3 in the acetate-blocked exposure but nearly full retention of $^{32}$P labeling. Intensities are quantified in Table I. $i$, $ii$, $iii$ denote bands whose intensity is quantified in Table I. B, decreasing amounts but equal volumes of each radioisotope (starting intensity, ~5 nCi; bottom line) were spotted onto Whatman polyethyleneimine-impregnated p81 paper. After drying, the paper was imaged for 24 h without (left) or with (right) two acetate sheets placed between the paper and the screen; the difference in intensities at this time was about 1:3 ($^{32}$P/$^{33}$P). Hence 3 times more $^{33}$P isotope compared with $^{32}$P isotope needs to be used for in vivo labeling. Note again that there is only slight attenuation of $^{32}$P intensity with acetate sheets in place. Intensities are quantified in Table I. dil, dilution.
**33P/32P Differential Gel Electrophoresis**

**Table I**

Quantitation of intensities shown in Fig. 1

Raw phosphorimages were imported into NIH Image, and the intensity of the three bands indicated in Fig. 1A (i, ii, and iii) was quantified on a scale of 0–255. Note the lack of effect of the acetate sheets on the intensity of the 32P-labeled proteins (compare lane 5 with 2), the loss of 33P values in the presence of acetate sheets (compare lane 4 with 1 and lane 6 with 3), and the linearity of phosphorimaging, indicated as the restoration of the values measured for lane 3 by adding the values measured in lanes 1 and 6 minus the values in lane 4 (column 7). A similar effect was found when the intensity of the spots shown in Fig. 1B was quantified.

| Cycle | 0 acetate sheets | 2 acetate sheets | Lanes 1 + 6 − 4 |
|-------|-----------------|-----------------|----------------|
|       | 33P | 32P | 33P + 32P | 33P | 32P | 33P + 32P | Lanes 1 + 6 − 4 |
| Fig. 1A |     |     |           |     |     |           |               |
| i    | 50  | 139 | 178       | 5   | 138 | 140       | 185           |
| ii   | 56  | 158 | 191       | 7   | 152 | 151       | 200           |
| iii  | 33P | 32P | 163       | 3   | 126 | 131       | 171           |
| Fig. 1B |     |     |           |     |     |           |               |
| 1:4  | 7   | 40  | 0         | 0   | 39  |           |               |
| 1:3  | 14  | 61  | 0         | 0   | 60  |           |               |
| 1:2  | 20  | 90  | 0         | 0   | 89  |           |               |
| 1:1  | 37  | 133 | 1         | 3   | 132 |           |               |
| 1    | 55  | 195 |           | 4   | 191 |           |               |

in Table I. It can be seen that 33P labeling detected during the first exposure (lane 1) was essentially completely eliminated when the gel was re-exposed using two acetate sheets (lane 3). However, near full retention of 32P signal was obtained in the single 33P-labeled sample (lanes 2 and 5) or in the mixed sample (lanes 3 and 6) without or with acetate. Moreover there was no interference between the 33P and 32P when imaged together as shown by the reconstitution of the combined values measured in the mixed sample (lane 3) when the value measured in the 33P-labeled sample (lane 1) was added to that of 32P captured under two acetate sheets (lane 5). With one acetate screen present, 33P radiation was still marginally detectable, whereas with three screens, the 32P bands had become more diffuse. Notably the 14C-markers were also screened out using the acetate sheets.

As the cells in Fig. 1A were labeled with equivalent amounts of radioactivity, the amount of 33P detected was relatively low compared with that of 32P. We therefore performed a quick test to ensure detection of equal amounts of radiation for both isotopes. For this purpose equal volumes of 33P and 32P were spotted onto polyethyleneimine-impregnated P81 paper at increasing dilutions. Fig. 1B shows that although theoretically the energy emitted from 33P is 6.8-fold less than that of 32P, practically we found that the intensity of 33P as detected by the phosphorimaging screen equalled that of 32P when the sample of 32P was diluted between 3–4-fold relative to that of 33P. Again detection of 33P (Fig. 1B, right) was completely eliminated by two acetate sheets, whereas that of 32P was hardly attenuated (see quantitation in Table I). Of course, the relative ratios of the two isotopes would have to be determined for each experiment as the half-life of 32P is 14 days, whereas that of 33P is 25.4 days. Using this “spot detection test,” the assay is quick and simple.

We next examined whether this method could be used to detect kinase-specific labeling of proteins in SCG neurons. To demonstrate first that we can detect the various phosphoproteins of stathmin in the neurons, SCG neurons were 32P-labeled in the presence of CPTcAMP and NGF (as well as araC; see below), and proteins were separated by 2D electrophoresis. Fig. 2A shows the overall pattern of 32P labeling achieved (left) together with the Coomassie Blue-stained image of the gel (right). The various tiers of stathmin and the number of phosphates incorporated (P1–P4) are indicated in the rectangle using the notation described by Beretta et al. (4). The most prevalent forms on the Coomassie image are α0 (or N1, the nonphosphorylated form of stathmin) and α1, the first tier singly phosphorylated form of the protein. On the 32P-labeled gel, the N1 form is naturally absent, whereas all the other forms reported previously after in vitro phosphorylation with PKA and Cdc2 are present, namely the first tier proteins (α1, α2, and α3) aligning with P1, P2, and P3; the second tier spots in set 17 (α1, and α2) aligning with P2 and P3; and third tier spots in set 16 (α1, α2, and α3) aligning with P3 and P4, the most acidic phosphorylated spot. Minor β forms were also sometimes noted, but these did not appear in all gels. These spots are annotated in greater detail in Fig. 2, C and D, as explained below.

The underlying purpose of our study was to detect ERK targets that protect against araC-induced apoptosis. NGF maintains a sustained activation of ERK (10), which partially antagonizes the proapoptotic signal induced by araC (a p53-dependent type of apoptosis (17)). Protection by ERK is thus suppressed by the MEK inhibitors PD98059 (75 μM) or U0126 (10 μM) (11, 17–19). Stathmin in this context was used to demonstrate that ERK inhibition by the drug had occurred. It is important to note that to detect ERK-dependent labeled phosphoproteins using simultaneous analysis of 33P and 32P,
the inhibitor must be used in conjunction with $^{32}$P as the image of the $^{33}$P-labeled proteins that were phosphorylated by ERK will be eliminated by the acetate sheets, leaving to appear only those (32P-labeled) spots that were phosphorylated independently of ERK. Accordingly one set of SCG neurons was labeled with $^{33}$P (about 0.5 mCi) in the absence of U0126 to label the entire phosphoproteome cohort, whereas the other set was labeled with $^{32}$P (about 0.17 mCi) in the presence of U0126. In this case, a 3-fold higher amount of $^{33}$P radionuclide was added compared with $^{32}$P to equalize loading. Equalizing the signal of the two isotopes by varying the amount of radioactivity of $^{32}$P and $^{33}$P used to label the cells.

**Fig. 2. Evidence for efficacy of the DIPPL method.** A, low power overview of a typical 2D gel on which a $^{32}$P-labeled sample of SCG neurons has been separated; on the left is the image collected from the phosphorimaging screen, and on the right is the Coomassie Blue-stained scanned digital image. The rectangle encloses the region where the various multiply phosphorylated stathmin forms are located, the labeling indicating the three tiers identified in the text. B, stathmin phosphorylation. Serines 16 and 63 are predicted to be phosphorylated due to CPTcAMP activation of PKA, whereas serine 25 is predicted to be the major residue phosphorylated by ERK. Serine 38 is also phosphorylated by ERK, but it is not clear whether this is mediated by ERK in the neurons. C and D, schematics of the phosphostathmin forms traced from the adjacent image identified according to Beretta et al. (4). C shows the $\alpha$ forms with the arrows showing the conversion pattern expected in the presence of U0126. D traces the pattern of the minor $\beta$ forms observed with the respective expected conversion pattern. E and F, comparison of the stathmin pattern obtained when two samples are independently labeled with $^{32}$P and proteins are separated on two independent 2D gels. E, neurons were labeled in the presence of CPTcAMP and araC for 3 h, and then DMSO (U0126 solvent) was added for 0.5 h after which NGF was added for an additional 3 h. F, neurons were labeled in the presence of CPTcAMP and araC for 3 h, and then U0126 was added for 0.5 h after which NGF was added for 3 h. G and H, comparison of the stathmin pattern obtained when two samples are independently labeled as in E and F except that $^{33}$P was used to label cells in the absence of U0126 and $^{32}$P was used to label cells in the presence of U0126. Samples were mixed and run on a single 2D gel. G, no acetate sheets (both radioisotopes imaged). H, re-exposure with two intervening acetate sheets. Note the similarity between E and G on the one hand and F and H on the other hand. Intensities are quantified in Table II.
most are those labeled at serine 25 (9). The schematic in Fig.
rylation sites in stathmin, two expected to be induced by (4).
expression of ERK-dependent phosphorylation, and its successful in-
P53-induced apoptosis, we newly demonstrated that phos-
horus-labeled stathmin can be used to monitor and verify
ERK-dependent phosphorylation, and its successful inhibi-
by U0126, occurred in SCG neurons.

The schematic in Fig. 2B shows the four possible phospho-
ylation sites in stathmin, two expected to be induced by CPTcAMP and two expected to be induced by NGF via ERK activa-
tion. The spots whose intensities are predicted to alter most are those labeled at serine 25 (9). The schematic in Fig.
2C (tracing the pattern in E and G of the α forms of stathmin) indicates the seven major spots detected, while the arrows indicate the conversion pattern expected if serine 25 phos-
phorylation is attenuated in the presence of U0126; generally,
tier 2 and 3 spots should disappear (the same pattern is predicted irrespective of whether serine 38 phosphorylation is eliminated or not (4)). Fig. 2D indicates the location of some of the β form spots detected and their expected conversion pattern; indeed, as expected, these were minor spots.

As predicted, in both the single 32P-labeled sample treated with U0126 (Fig. 2F) and the mixed 33P/32P sample exposed with two acetate sheets interposed (Fig. 2H), phosphorylation of the two major tier 2 and 3 spots (sets 16 and 17) was eliminated by the presence of U0126 (see Table II for quantifi-
cation). The intensity of the two slowly migrating minor β spots was also reduced, consistent with these being similarly regulated (4). Thus, comparing across the two types of label-
ing, it is clear that the acetate sheets successfully masked the 32P-labeled proteins (Fig. 2, compare H and G, and Table II). The 32P-labeled protein pattern is slightly more diffuse with the acetate sheets present than the images collected from the equivalently treated 33P labeled sample because the gel was imaged sequentially, and there was some decay of 32P during the time required to collect the 33P image. Increasing and adjusting the amount of 33P and 32P the cells incorporate, thereby shortening the time required for exposure, will com-

| Ratios | α1 | α2 | α3 | α11 | α12 | α21 | α22 |
|--------|----|----|----|-----|-----|-----|-----|
| 32P/32P (F/E) | 1   |   |    |     |     |     |     |
| 32P/(33P + 32P) (H/G) | 1   | 0.86 |    | 0.96 | 0.41 | 0.35 | 0.10 |

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