construct. The methodology that combines particle-bombardment transfection procedures and organotypic slice culture techniques might be a promising approach (1,5,6). Therefore, we focused on applying our biolistic device to neural tissues.

The dimensions of the apparatus and the intensity of the electric discharge have to be adjusted for each target tissue type. For rat cerebral cortex slices, we use the following values. The internal dimension of the discharge chamber is 13 mm in diameter and 15 mm in depth. The discharge electrode is a 2-mm wide, 35-µm thick and 10-mm long copper wire. A 200-V, 12,000-µF capacitor is used. The carrier sheet is a 20-mm square, 50-µm thick polyimide film. The screen locates 10 mm below the chamber. The target tissue locates 10 mm below the screen.

An example of the results of transfection experiments is shown in Figure 2. Cells that express β-galactosidase are stained blue. The number of cells transfected in the slice illustrates the level of transfection achieved by biolistics. Cell body and horizontally and/or vertically running long processes of each cell were clearly observed. When the slice was placed on the membrane, several dissociated cells were also placed on the membrane. Some of these dissociated cells were stained blue. This demonstrates that our biolistic device also works for dissociated cells.

Particle bombardment is used to transfect a broad range of cell and tissue types including plant meristems, insect embryos, fish embryos, bacteria, fungi, animal tissue slices and animal tissue cultured cells. In trying our biolistic device on these applications, the following adjustments or trade-offs should be considered. The diameter of the discharge chamber and the distance between the screen and the target tissue should be designed to cover the size of the target tissue. The diameter of the particles should be much smaller than the size of the target cells to avoid cell damage. The density of the particles on the target should be high enough that many cells receive particles, but low enough to avoid the situation where multiple particles converge to the same target cell and cause cell damage.

The velocity of the particles should be adjusted so that the particles penetrate the tissue surface and stop in the tissue. Velocity of the particles may be a complicated function of the dimension of the discharge chamber, distance to the target and the electric discharge power, and is difficult to estimate theoretically. It is practical to adjust the velocity empirically by varying the electric discharge power. The electric discharge power is proportional to the product of the capacity of the capacitor and the square of the applied voltage. Electric discharge power is easily and continuously controlled by changing the capacity of the capacitor and/or the applied voltage. The ease of the power control will be an advantage of the electric-discharge particle accelerator.

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Shigeru Takagi and Minoru Kimura1
NTT Basic Research Laboratories
Kanagawa
1School of Medicine
Tokai University
Isehara, Japan

Modification of an In Situ Renaturation Method for Analysis of Protein Kinase Activity with Multiple Substrates

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Renaturation of enzyme subunits in situ following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of protein preparations is a powerful tool for analyzing protein kinases (2,4). The technique allows for evaluating the efficacy of putative in vivo and model protein kinase substrates, divalent cation requirements and nucleotide specificity of several different protein kinases in a single preparation based on their subunit molecular weight (MWsub). We have found this technique useful for examining changes in protein kinase activities in response to anaerobic stress in the rice weed, Echinochloa phyllogon L. Beauv. More specifically, we are using it to study the role of phosphorylation of enolase during anaerobiosis.

One drawback with the current technique is the requirement for relatively large quantities of protein substrate (20–40 mg) to be incorporated into the
In our experience, it is preferable to run standard-size gels rather than minigels. The greater separation between the protein kinases in standard-size gels allows better resolution after autoradiography. With model substrates such as casein, phospholipase D, histone and protamine, larger quantities are less problematic because the substrates can be obtained commercially at reasonable cost. When evaluating putative in vivo substrates, however, obtaining the necessary amount of purified protein (20–40 mg) required for one standard-size PROTEAN® II xi Slab Gel (Bio-Rad, Hercules, CA, USA) may be difficult with respect to cost and time requirements when tissue amounts are limited. We encountered both kinds of problems when studying enolase phosphorylation in response to anaerobic stress in *E. phyllopogon*. Initially, we made use of commercially available sources of rabbit and yeast enolases. When we compared these sources with enolase purified from *E. phyllopogon*, however, we found different sets of protein kinases interacting with the different enolases (data not shown). Consequently, we prefer to use enolase purified from *E. phyllopogon* for our studies.

Figure 1. Modified in situ renaturation apparatus for simultaneous analysis of phosphorylation substrates. Photograph (A) and diagram (B) of a PROTEAN II xi Slab Gel System modified to run four modular gels in parallel. The resolving gel (0.75 mm thick) is divided into four modular gels by adding three spacers trimmed to 13.7 × 0.6 cm (slightly taller than the height of the resolving gel and the width of one well). The spacers are sealed in place with vacuum grease, allowing each minigel to be filled with an SDS-PAGE gel matrix (13.9% T, 2.7% C) containing 0.5 mg/mL of different protein substrates (e.g., enolase, casein, phospholipase D, histone, protamine) without cross-contamination.
Purification of enolase from *E. phyllopagon* is a four-step process involving ethanol precipitation, ammonium sulfate fractionation, Q Sepharose® (Pharmacia Biotech, Piscataway, NJ, USA) anion-exchange chromatography and gel filtration that requires three to four days to complete and yields only 0.25–0.50 mg protein from 10 g of shoot tissue (6). Large quantities of plant material are not readily available, and the low yields of purified enolase caused us to look for ways to reduce the amount of enolase protein required for in situ renaturation experiments.

We have modified our slab gel system (16 × 20 cm, 0.75 mm thick) to require less substrate while retaining the resolution of the longer gel (Figure 1). We divided the resolving gel into four modular gels by adding three spacers trimmed to 13.7 cm × 0.6 cm × 0.75 mm (i.e., slightly taller than the resolving gel and the width of one well). The spacers were sealed in place with vacuum grease, allowing each modular gel to be filled with SDS-PAGE gel matrix (13.9% T, 2.7% C) containing 0.5 to be filled with SDS-PAGE gel matrix and the width of one well). The cut edges of the gels served as initiating points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tearing and necessitated points for tuning. By reducing both gel volume and the concentration of protein substrate to 0.5 mg/mL, only 3.5 mg of enolase per gel were required. Substrate concentrations ranging from 0.2 to 1.0 mg/mL were evaluated, and 0.5 mg/mL was judged as the minimum concentration to yield satisfactory detection of protein kinases. After polymerization of the resolving gel, a common stacking gel was poured, and proteins in crude extracts were resolved by standard SDS-PAGE. Following electrophoresis, SDS was removed from the gels by washing 5 times in 50 mM Tris-HCl (pH 7.6) for 15 min. In the examples shown here (Figure 2), phosphorylation was carried out by incubating individual gels in 40 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 10 mM CaCl₂, 10 mM MnCl₂ and 250 μCi [γ-³²P]ATP for 2 h at 25°C. Gels were washed three times with 40 mM Tris-HCl (pH 7.6), 1 mM EDTA, 2.5 mM Na⁺ pyrophosphate and 5% (wt/vol) Dowex® 2 × 8-50 resin (Fluka, Hauppauge, NY, USA) for 1 h to remove unincorporated ³²P. The gels were briefly rinsed with deionized water and either fixed with three washes in 10% (wt/vol) trichloroacetic acid (TCA) at 70°C for 1 h or treated with 1 M KOH at 60°C for 1 h to dephosphorylate some ser/thr phosphoproteins and fixed with two washes in 10% (vol/vol) acetic acid at 60°C for 1 h (1). The gels were placed on plastic supports, sealed in plastic wrap and exposed directly to Fuji X-ray film at 4°C for 1–3 days.

Figure 2 shows the results of a typical in situ renaturation electrophoretic run in which the standard gel was divided into four modular gels in parallel. Adjacent modular gels contained either casein (Figure 2, A and B) or phospholipase D (Figure 2, C and D). With casein as substrate, two polypeptides of 15 and 45 kDa were detected. Phosphorylation of the 15-kDa protein was enhanced in shoots of anaerobically grown seedlings, whereas the activity of the 45-kDa protein kinase was insensitive to the anaerobic treatment (Figure 2A). Treatment of the duplicate modular gel with potassium hydroxide dephosphorylated both proteins, indicating that phosphorylation occurs at ser/thr residues (Figure 2B). The size of the 45-kDa phosphoprotein is within the typical range for ser/thr protein kinases, and dephosphorylation with potassium hydroxide supports this conclusion. The 15-kDa phosphoprotein, however, is too small to contain the catalytic subunit typical of ser/thr protein kinases and may reflect the activity of nucleoside diphosphate kinase (5). With phospholipase D incorporated into the gel matrix, protein kinases with MWsub of 56 and 62 kDa were stimulated by anoxia, those of 36 and 49 kDa were repressed by anaerobic treatment, and a protein kinase MWsub of 54 kDa exhibited similar activities in both treatments (Figure 2C). Potassium hydroxide treatment did not dephosphorylate any of the labeled proteins, although the amount of phosphorylation of the 49- and 62-kDa polypeptides was reduced (Figure 2D). Since potassium hydroxide does not dephosphorylate all ser/thr phosphoproteins, we cannot definitively conclude whether these are ser/thr or tyr protein kinases from these results.

From a technical point of view, it is important to note that no cross-contamination between adjacent modular gels containing different substrates (Figure 2, B vs. C) was observed. The MWsub of protein kinases acting on casein (Figure 2, A and B) was completely different from those acting on phospholipase D (Figure 2, C and D). Thus, we can survey up to four protein kinase substrates in one electrophoretic run with confidence that the protein kinases are specific to the intended substrate and not the result of protein substrates leaking from adjacent modular gels.

The technique of dividing the larger gel into modular gels is also advantageous when duplicate gels are required. Previously, we ran duplicate lanes and cut the gel for differential processing. The cut edges of the gels served as initiating points for tearing and necessitated very gentle handling of the gels during the high number of washes required in the protocol. The tendency of the gel to tear was exacerbated when SDS was removed from the gels and they became more fragile. The polymerized surface at the edges of the modular gels reduced the likelihood of tearing and made the gels easier to handle. Generation of multiple modular gels that contain the same protein substrate in one

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**Figure 2. In situ renaturation of protein kinase activity.** Protein extracts from shoots of *E. phyllopagon* seedlings grown aerobically (O₂) or anaerobically (N₂) were run in four modular gels in parallel. Panels A and B contained casein incorporated into the gel matrix, whereas Panels C and D contained phospholipase D. Following electrophoresis, SDS was removed from the gels and phosphorylation carried out in vitro. Parallel modular gels were treated with KOH following phosphorylation, and the resultant loss of ³²P in specific polypeptides suggests the presence of ser/thr kinases (Panels B and D).
electrophoretic run permits convenient side-by-side comparisons among several different kinase reaction conditions (e.g., the divalent cation \([\text{Ca}^{2+}, \text{Mg}^{2+} \text{or Mn}^{2+}]\) requirements or various reaction pHs) following electrophoresis.

In situ renaturation detection of protein kinase activity is a time-consuming and fairly expensive process. The modifications presented here allow for the simultaneous processing of four different sets of protein kinases or generation of multiple gels to assess up to four different protein kinase reaction conditions in a single electrophoretic run. The resolving power of larger slab gels is retained while reducing the amount of protein substrates required by nearly 90%. Thus, the modular gel technique described here provides greater experimental flexibility and increased efficiency, leading to new levels of resolution compared to standard full-sized gels or minigels.

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Theodore C. Fox and Mary E. Rumpho
Texas A&M University
College Station, TX, USA