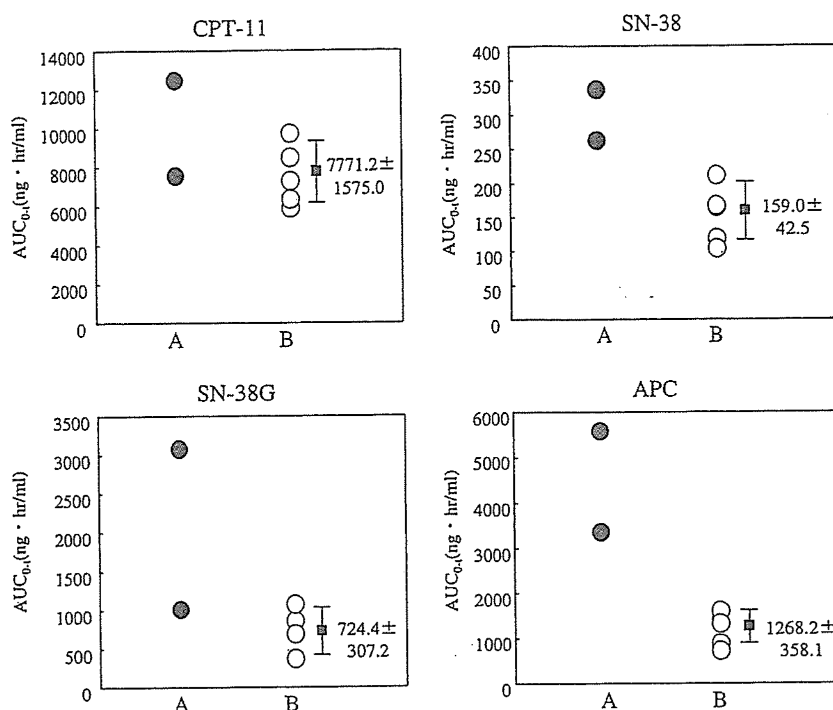

Fig. 2 Area under the concentration versus time curve for irinotecan and metabolites in patients with biliary drainage (A, $n = 2$) and without drainage (B, $n = 5$). The values are expressed as the mean ± SD

Table 5 Pharmacokinetic parameters after single administration of irinotecan at a dose of 100 mg/m^2 ($n = 7$)

		C_{max} (ng/ml)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{0-t} (ngh/ml)	CL (l/h m^2)
Irinotecan	A	1,188.5, 1,997.6	1.6, 1.5	7.8, 8.2	7,762, 12,692	11.8, 7.1
	B	1,701.0 ± 348.3	1.5 ± 0.1	7.7 ± 0.9	7,771.2 ± 1,575.0	12.4 ± 2.5
SN-38	A	25.5, 26.2	2.1, 1.5	14.7, 9.9	268, 342	–
	B	17.5 ± 3.8	2.3 ± 0.8	30.2 ± 27.6	159.0 ± 42.5	
SN-38G	A	81.3, 207.2	3.6, 2.0	10.8, 12.5	1,063, 3,130	–
	B	78.8 ± 34.1	2.2 ± 0.2	21.6 ± 13.2	724.4 ± 307.2	
APC	A	309.2, 359.3	2.6, 5.5	7.0, 9.5	3,441, 5,673	–
	B	116.6 ± 39.7	3.0 ± 0.6	8.8 ± 0.7	1,268.2 ± 358.1	

A Patients with biliary drainage $n = 2$

B Patients without biliary drainage (parameters are represented as the mean ± SD) $n = 5$

episodes before onset. Although our study indicated that weekly irinotecan administration would be tolerable in patients with metastatic pancreatic cancer, careful observation is required during the treatment period, since pancreatic cancer patients tend to suffer various tumor-related complications and easily take a turn to the worse because of tumor progression.

There are two studies of single-agent irinotecan that assessed efficacy and toxicity against pancreatic cancer [14, 22]. Sakata et al. [14] studied irinotecan at a dose of 100 or 150 mg/m^2 administered weekly or bi-weekly to previously treated or untreated patients with pancreatic cancer in Japan. Although 57 of 61 enrolled patients were assessable, only 4 patients (7.0%) showed a PR. This study included 28 patients (49.1%) with poor performance status of 2–3 and 22 patients (38.6%) with prior chemotherapy, and no patient showed a PR in these patients with poor performance status or prior chemotherapy. Wagener et al. [22] demonstrated that irinotecan at a dose of 350 mg/m^2 administered every 3 weeks to chemo-naïve pancreatic cancer patients with performance status of ≤ 2, achieved a PR in 3 of 32 assessable patients (9.4%) with an median overall survival of 5.2 months. Although precise reason for the discrepant response rates between our study and the other two studies is unclear, patient background may be one possible explanation because only chemo-naïve patients with good performance status were entered into our study (89.2% of our patients had good KPS of ≥ 90).

For the purpose of the improvement on response rate and prognosis, several studies of combination therapy have been conducted in patients with pancreatic cancer. With regard to irinotecan with gemcitabine, an

encouraging activity, response rates between 20.0 and 24.7% and median overall survival between 5.7 and 7 months, have been reported in two phase II studies [11, 18]. However, survival benefit of this combination therapy was not shown in a phase III study [12], in which, 360 patients were randomized to treatment with a combination of gemcitabine 1,000 mg/m^2 followed by irinotecan 100 mg/m^2 given on days 1 and 8 of a 3-week cycle versus gemcitabine monotherapy. The response rate for the combination therapy was higher at 16.1% compared with 4.4% for gemcitabine alone, but there was no difference in median overall survival (6.3 vs. 6.6 months). However, several clinical studies have recently indicated that irinotecan-based chemotherapy seemed to be an effective treatment for advanced pancreatic cancer after gemcitabine failure: irinotecan–raltitrexed combination demonstrated overall response rate of 16% (3/19) in patients with gemcitabine-pretreated pancreatic cancer [21], and Cantore et al. [3] reported that irinotecan plus oxaliplatin showed response rate of 10% (3/30) with a clinical benefit response of 20% (6/30) for patients with advanced pancreatic cancer after gemcitabine failure.

Because biliary excretion is a major elimination pathway for irinotecan and its metabolites, we investigated the impact of biliary drainage on the pharmacokinetics for this agent. Our results suggested that patients with biliary drainage tended to have higher area under the concentration versus time curve of irinotecan and metabolites compared with patients without biliary drainage. Meyerhardt et al. [10] reported that modest elevation of bilirubin (1.0–1.5 mg/dl) is associated with increased grade 3 to 4 neutropenia in patients treated with irinotecan. The fact that the two patients with biliary drainage in the current study had slight elevation of baseline serum bilirubin level (1.4 and 1.7 mg/dl) might influence pharmacokinetics for irinotecan. Although no severe hematological or non-hematologic toxicities appeared in these two patients, careful observation may be required when treating patients with biliary drainage.

In conclusion, single-agent irinotecan showed a substantial antitumor activity for patients with metastatic pancreatic cancer, rendering a 27.0% response rate. The toxicity with this schedule appears manageable, though it must be monitored carefully.

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Application of highly sensitive fluorescent dyes (CyDye DIGE Fluor saturation dyes) to laser microdissection and two-dimensional difference gel electrophoresis (2D-DIGE) for cancer proteomics

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Proteome data combined with histopathological information provides important, novel clues for understanding cancer biology and reveals candidates for tumor markers and therapeutic targets. We have established an application of a highly sensitive fluorescent dye (CyDye DIGE Fluor saturation dye), developed for two-dimensional difference gel electrophoresis (2D-DIGE), to the labeling of proteins extracted from laser microdissected tissues. The use of the dye dramatically decreases the protein amount and, in turn, the number of cells required for 2D-DIGE; the cells obtained from a 1 mm^2 area of an 8–12 μm thick tissue section generate up to 5,000 protein spots in a large-format 2D gel. This protocol allows the execution of large-scale proteomics in a more efficient, accurate and reproducible way. The protocol can be used to examine a single sample in 5 d or to examine hundreds of samples in large-scale proteomics.

INTRODUCTION

Laser microdissection in cancer research

Tumor tissues consist of histologically heterogeneous populations of cells. They include multiple populations of tumor cells with different degrees of histological differentiation and mitotic status, and also contain various types of noncancerous cells. Individual cell populations presumably have a distinct molecular background at the DNA, RNA and protein levels. Thus, when tumor tissues are homogenized and treated as a whole during sample preparation, the results of subsequent experiments can reflect two factors: the molecular contents of the cells, and the ratio of the different cell populations. Further experiments employing other techniques, such as immunohistochemistry or *in situ* hybridization, might not distinguish which of the two factors contributed more dominantly to the results of the experiments. In addition, as the ratio of the different cell populations varies between individual cases, it may be difficult to obtain consistent results from multiple samples when the tissues are homogenized as a whole. Cancer-associated vasculature should be taken into account in the sampling process to distinguish the plasma proteins from the proteins that are to be released but are still present within the cells. Overall, to obtain straightforward and consistent conclusions, specific populations of cells should be isolated before DNA, RNA and protein extraction.

Laser microdissection is a means of isolating a specific population of cells; the target cells are directly recovered under microscopic observation to the exclusion of other cell types and are subsequently subjected to experiments to study DNA, RNA and protein contents. Laser microdissection is an established method, and several different types of well-designed devices are commercially available[1]. However, recovering enough cells to obtain adequate amounts of DNA, RNA and protein may sometimes be labor-intensive and time-consuming with some of these procedures to a degree that makes laser microdissection impractical. In the case of DNA and RNA studies, this problem may be partially solved by amplifying the DNA and the reverse-transcribed DNA from RNA respectively[2]. In protein studies, however, as proteins and peptides cannot be amplified, the sensitivity of the protein detection method determines the minimal amount of protein and, in turn, the minimal number of cells required. Thus, the combination of a highly sensitive protein detection method, equivalent to PCR in molecular biology, with the application of laser microdissection is critically important to the study of protein contents.

Laser microdissection with 2D-PAGE and 2D-DIGE

Laser microdissection has been combined with two-dimensional (2D)-PAGE in cancer proteomics, in which the overall features of protein expression are correlated with cancer phenotypes to identify the molecular background of cancer biology[3]. 2D-PAGE is a high-resolution (delta pI = 0.001 by narrow IPGs with 24 cm separation distance), wide-coverage (pH range 2.5–12), reproducible and high-throughput method (with difference gel electrophoresis (DIGE) technology, see below) that generates quantitative proteomic data for hundreds to thousands of proteins[4,5]. One of the unique advantages of 2D-PAGE is that the acquired proteomic data can be stored as 2D images and utilized for post-planned analysis[6]. Furthermore, mass spectrometric protein identification directly from the protein spots has made 2D-PAGE an indispensable proteomics method. The combination of laser microdissection with 2D-PAGE would therefore appear to be a very powerful and promising tool for cancer proteomics. However, the low sensitivity of protein detection methods in 2D-PAGE had so far limited the application of laser microdissection; several hours to days of laser microdissection were required to recover enough cells for 2D-PAGE. This throughput is far from the efficiency required of cancer proteomics, in which hundreds of clinical specimens are compared

to identify the proteins corresponding to certain clinico-pathological parameters. Even with silver staining[3,7], which is the most sensitive colorimetric protein detection method for 2D-PAGE, the number of samples examined was not sufficient to obtain statistically valid conclusions, presumably because of the prohibitively labor-intensive task of recovering the cells. In addition, probably because of artificial damage to the cells during the time-consuming experiments, the quality of the gels and, in turn, the number of reproducibly detected protein spots did not reach the level expected of or desired in a comprehensive expression study. Metabolic labeling of the cells using ^{35}S-methionine is a highly sensitive method for 2D-PAGE[8], but cannot be applied to laser-microdissection because laser microdissected tissues have lost the ability to physiologically incorporate amino acids. Although a protocol to label extracted proteins with ^{125}I and ^{131}I has been applied to laser microdissected tissues, its sensitivity was still limited, and pooled, instead of individual, samples were used in 2D-PAGE[9]. The use of pooled samples could underestimate the variations of protein contents between individual cases and might not result in statistically valid conclusions. Thus, even with the use of the best existing protein-detection methods, the combination of laser microdissection with 2D-PAGE appeared to be impractical for cancer proteomics.

In 2003, we reported a novel application of highly sensitive fluorescent dye (CyDye DIGE Fluor saturation dye) to the labeling of proteins extracted from small numbers of cells recovered by laser microdissection[10]. We found that the use of this dye dramatically decreased the amount of protein and, in turn, the number of cells required for 2D-PAGE. In our protocol, the number of cells in a 1 mm^2 area of a 10 μm thick section was sufficient to generate one 2D gel image, and therefore laser microdissection required only less than 1 h per sample. We also confirmed that the proteins labeled with CyDye DIGE Fluor saturation dye were suitable for mass spectrometric protein identification[10]. The multiple use of laser scanners for the detection of the labeled proteins enabled very-high-throughput 2D-PAGE, leading to a large-scale proteome study (see below). With the application of CyDye DIGE Fluor saturation dye, laser microdissection has thus become a practical tool for cancer proteomics.

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The CyDye DIGE Fluor saturation dye was developed for 2D difference gel electrophoresis (2D-DIGE). In 2D-DIGE, the protein samples to be compared are labeled with mass- and charge-matched spectrally resolvable fluorescent dyes, mixed together and co-separated by 2D-PAGE in the same gel. Multiplex images are obtained by laser scanning the gel at the appropriate wavelengths. The intensity of protein spots can be compared between different samples, without being affected by gel-to-gel variations. Two types of fluorescent dyes are presently available for 2D-DIGE: CyDye DIGE Fluor minimal dye, and CyDye DIGE Fluor saturation dye. The former labels a small portion of lysine residues[11]; its sensitivity is similar to that of silver staining, and labeling with it does not change the electrophoretic mobility of the protein. By contrast, the latter labels all reduced cystein residues of protein[12]; as a consequence, the dye affects the electrophoretic mobility of the protein and has 100–200-times higher sensitivity than CyDye DIGE Fluor minimal dye. CyDye DIGE Fluor minimal dye has previously been applied to the labeling of proteins recovered from laser microdissected tissues[13,14]. However, due to the limited sensitivity of available fluorescent dyes, a very high number of cells—ranging from 30,000 (ref. 13) to 250,000 (ref. 14) cells—and, consequently, daunting labor-intensive laser microdissection tasks were required for 2D-DIGE. The use of CyDye DIGE Fluor saturation dye, which has 100–200-times higher sensitivity than CyDye DIGE Fluor minimal dye[12], made experiments combining laser microdissection with 2D-DIGE substantially more feasible.

2D-DIGE has a unique advantage over the classical 2D-PAGE in cancer proteomics—where reproducible and quantitative protein expression profiling of multiple samples is required—in that it allows the inclusion in all gels of an internal control sample labeled with a fluorescent dye different from that of individual samples. By normalizing the 2D image of the individual sample with that of the internal control sample, gel-to-gel variations can be canceled out even across multiple gels[15,16]. The use of such an internal control sample can solve a generic problem of 2D-PAGE and improve its reproducibility and quantitativity. The wide dynamic range of fluorescent dyes also contributes to the performance of quantitative comparisons. The other notable advantage of 2D-DIGE is its high throughput. In classical 2D-PAGE, colorimetric methods such as silver staining and Coomasie Briliant Blue have been used to detect protein spots. Because the procedure for gel-staining is labor intensive and time consuming, it is not practical to run many large-format 2D gels simultaneously[5]. In contrast, in 2D-DIGE, where protein spots to be detected are labeled with a fluorescent dye, multiplex images are obtained from a large-format 2D gel within 1 h by laser scanning. Therefore, by being equipped with multiple electrophoresis devices and laser scanners, we can examine large clinical sample sets in a less labor-intensive way and in a relatively short time. Overall, 2D-DIGE is a very powerful tool for differential proteomics in terms of its reproducibility, sensitivity (both in the range of proteins detected and their accurate quantification) and high throughput, as a more advanced version of 2D-PAGE.

The use of large-format 2D gels is critical for 2D-DIGE of laser microdissected tissues. A typical 2D-DIGE using conventional electrophoresis devices such as the Ettan Dalt*twelve* separation unit (GE Healthcare Bio-sciences, cat. no. 80-6466-46) generates a 2D image with up to 2,000 protein spots. Taking into account that there are between 20,000 and 25,000 nonredundant proteins in the human proteome[17], it is clear that more protein spots should be examined to understand cancer biology. Protein fractionation prior to 2D-DIGE is a popular method used to enrich the low-quantity proteins and increase the number of protein spots detected[18–21]; still, the amount of such proteins from laser microdissected tissues is very small and a large portion may disappear during the multi-step procedures. Narrow pI range gels have also been used to dramatically increase the number of protein spots detected by improving the resolution[4], especially when combined with a prefractionation method[22]. However, to uncover a wide pI range, the combination of different pI range gels is required, which, again, multiplies the number of gels, the amount of proteins and the number of cells required. Therefore, the use of narrow pI range gels may be considered more suitable for the comprehensive study of a small number of samples. Instead, we propose the use of a large-format 2D gel, 24 cm Immobiline DryStrip for the first-dimension separation and SDS-PAGE with 37 cm distance for the second-dimension separation, for initial and routine comprehensive proteomic studies using laser microdissected tissues. The use of the long Immobiline DryStrip easily generates high-resolution and reproducible isoelectric

focusing[4]. In large-format 2D gels, low-intensity protein spots that are often shadowed by neighboring high-intensity ones in small 2D gels are visible, and not-well-focused protein spots are focused well following long-distance separation[23]. As a consequence, more protein spots are observed in large-format 2D gels for the same amount of protein and number of cells. The use of gradient gels also contributes to the increase in the number of spots detected by facilitating the effective use of limited gel area. Higher resolution in large-format gradient gels leads to more accurate spot recognition by the image analysis software. Spot detection by the image software may be problematic in areas overcrowded with protein spots; multiple spots are often combined into a smaller number of spots, while single spots may be artificially recognized as several spots. These problems may be resolved to some extent with the use of high-resolution separation of spots. A large-format 2D gel, albeit combined with silver staining, has been used for extensive proteomic studies[24]. In general, colorimetric spot detection using large-size gels requires very careful manipulation, a large space and experienced operators, because large-format 2D gels are very fragile and easily torn during staining procedures. However, in 2D-DIGE, the strength of the gel does not matter because the gel is directly scanned by laser while it remains between low-fluorescent glass plates, and gels with a size of up to the scan area of the laser scanner can be examined more efficiently. The proposed gel size and device are adjusted to those of the Typhoon (GE Healthcare Bio-sciences), a popular laser scanner. Therefore, the use of the large-format 2D gel proposed here is most suitable for large-scale proteomic studies using laser microdissected tissues and 2D-DIGE.

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Mass spectrometric identification of proteins labeled with CyDye DIGE Fluor saturation dye

In our protocol, protein identification is achieved by mass spectrometry using the proteins recovered from the preparative gel containing the higher amount of protein[10]. In the following protocol, an experiment using 100 μg of protein sample is demonstrated as an example. Target protein spots are identified on the preparative gel by matching the images of analytical and preparative gels. The protein sample for the preparative gel is prepared from laser microdissected tissue samples or, alternatively, tissue homogenates or cultured cells. CyDye DIGE Fluor saturation dye labels all reduced cysteine residues and, as a consequence, change the electrophoretic mobility of labeled proteins. Thus, the proteins for the preparative gel are all labeled with the CyDye DIGE Fluor saturation dye to match the image with that of the analytical gel. Although mass spectrometry continues to evolve every year and the way the mass spectrometry data can best be interpreted to identify proteins is still under discussion, the commercial mass spectrometric devices currently available seem to be of a level that is adequately advanced for protein identification. Therefore, the critical issues for protein identification at this point are the efficiency of protein extraction from the gel and the quality of the extracted proteins for mass spectrometric study. For mass spectrometric identification, the proteins in the gel are digested with a proteolytic enzyme and extracted with an organic solvent. This protocol, known as the in-gel digestion method, is popular and numerous variations have been reported. Because the most efficient in-gel digestion method should ideally differ between the individual target proteins and because there is no rule to optimize the protocol on the basis of the spot location on a 2D image, it may require significant experience to establish a protocol that can be used for most protein spots with the average optimal efficiency. Because the labeled peptides could have different properties in terms of recovery rate from the gel and ionization efficiency in the mass spectrometric study, some modifications are required for the existing in-gel digestion protocols. We have reported mass spectrometric protein identification for more than 1,000 protein spots labeled with CyDye DIGE Fluor saturation dye[10,18,25–29]. Sample sources included tissue specimens, cell lines and plasma. Over time, we continued to improve our protocol, examined an additional 1,600 protein spots and constructed our 2D-DIGE database. Thus the proposed protocol has been well-validated using a large number of labeled proteins with different physical characteristics. The following protocol uses multiple gel plugs in 96-well plates. Because one operator can manipulate two plates per day (192 spots), the protocol allows efficient protein identification.

Analysis of the proteome data obtained by 2D-DIGE

We propose the use of bioinformatics tools to analyze the 2D-DIGE data. Accurate sampling using laser microdissection and subsequent quantitative protein expression profiling using 2D-DIGE provide high-quality proteome data. It is of great interest to correlate the proteome data with clinico-pathological parameters and to select the most informative protein-expression pattern, namely the proteomic signature. Through the combined use of the bioinformatics tools, which include machine-learning methods, hierarchical clustering, principal-componentanalysis and cross-validation methods, we are able to create a class prediction model, which can be used to develop tumor markers to predict, among other parameters, early recurrence, nodal metastasis, response to therapy and patient outcome. This strategy has been proven to be effective in studies on DNA microarray data[30] and is applicable to 2D-PAGE data[31]. Large-scale proteomic data generated using 2D-DIGE have also provided a suitable resource for the use of bioinfomatics in cancer biology[28,29].

Versatility of the proposed protocol

In our laboratory, the protein-expression profiles of surgical specimens, including those from lung cancer[25], esophageal cancer[29], liver cancer and pancreatic cancer, have been examined in the past 5 years, combining laser microdissection with the use of CyDye DIGE Fluor saturation dye and 2D-DIGE. Thus, this strategy is potentially applicable for any type of solid tumor. In addition, because any type of diseased or normal tissue can be subjected to laser microdissection and 2D-DIGE, our application can be used in any proteomic research study, including studies on infectious diseases and cardiac diseases and in developmental biology. We have improved our original protocol[10] to increase the recovery rate of proteins, the number of protein spots and the reproducibility of the experiments. We found that the proposed protocol is flexible with regard to the characteristics of the samples and the purpose of the experiments. Since the publication of our report, the combined use of highly sensitive fluorescent dyes, laser microdissection and 2D-DIGE has been used, with some modifications, in several laboratories to study both normal and diseased tissues[32–35], and an application note regarding our protocol has been distributed by GE Healthcare Bio-sciences[36], suggesting an interest in our proposed protocol. The electrophoresis devices used in the protocol (GiantGelCaster and GiantGelRunner)

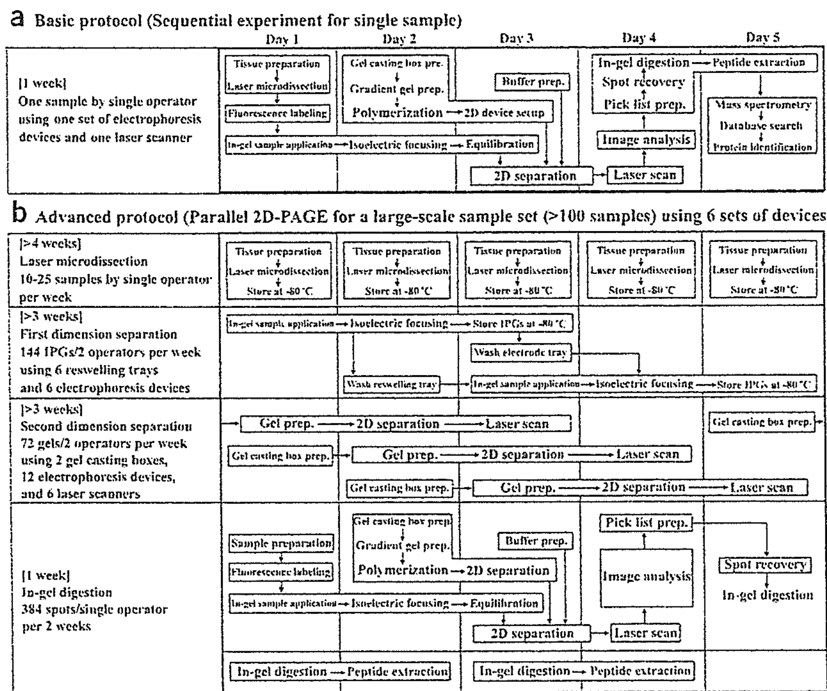

Figure 1 | Timetable showing the flow of the experimental protocol described, employing laser microdissection, 2D-DIGE and in-gel digestion in cancer proteomics. (**a**) Basic protocol (sequence of experiments for one sample). (**b**) Parallel experiments using multiple device sets (**Fig. 2**) can efficiently be conducted to study large-scale sample sets (more than 100 samples). The period for image analysis and the data-mining process, which depends largely on the number of samples, is not included in this figure.

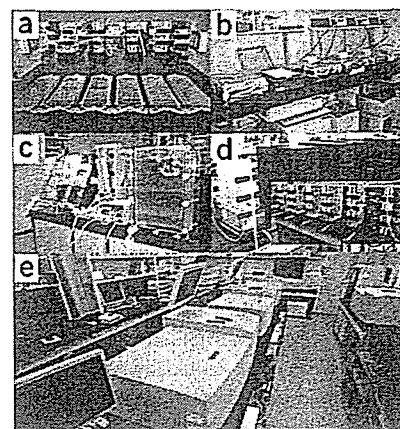

Figure 2 | Example of a laboratory equipped with the range of 2D-DIGE devices required for large-scale proteomics. (**a**) Six sets of Immobiline DryStrip Reswelling Trays, (**b**) Multiphor II electrophoresis system, (**c**) DALT Gradient Maker and two sets of GiantGelCasters, (**d**) Twenty-four sets of GiantGelRunners with dark boxes and (**e**) Typhoon Trio. Because 2D-DIGE allows parallel experiments, large-scale proteomic studies can be performed in a high-throughput way.

are larger versions of a gel casting device and an open-style SDS-PAGE device, and are commercially available. The procedure in this protocol can be easily applied using smaller devices such as the Ettan Dalt*twelve* Gel Caster (GE Healthcare Bio-sciences, cat. no. 80-6467-22) and the Ettan Dalt*twelve* separation unit (GE Healthcare Bio-sciences, cat. no. 80-6466-46). The extensively validated in-gel digestion protocol can be used as a standard protocol for labeled proteins. As far as the use of commercially available data-mining software, which was originally developed for the analysis of DNA microarray data, is concerned, we have demonstrated how to combine multiple DeCyder software files. Our protocol can be completed within 5 d for a single sample, and can be arranged to assay more than 100 samples by using multiple devices (**Figs. 1,2**). Thus, the proposed protocol is versatile in terms of sample types, laboratory conditions and purpose of experiments.

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MATERIALS

REAGENTS

- Surgical specimens **! CAUTION** The use of surgical material should be approved by the institution's ethics committee and written informed consent should be obtained from the donors.
- Optimum Cooling Temperature compound (OCT compound; Sakura Finetek, Tokyo, Japan; cat. no. 4583)
- Ethanol (Wako, Osaka, Japan; cat. no. 057-00451) **! CAUTION** Ethanol is flammable and a contact hazard. Wear gloves when handling.
- Formaldehyde (Wako; cat. no. 064-00406) **! CAUTION** Formaldehyde is flammable and a contact hazard. Wear gloves when handling.
- Mayer's hematoxylin solution (Muto Pure Chemicals, Tokyo, Japan; cat. no. 3000-2) **! CAUTION** Hematoxylin is flammable and a contact hazard. Wear gloves when handling.
- 0.5% eosin alcohol solution (Muto Pure Chemicals) **! CAUTION** Eosin is flammable and a contact hazard. Wear gloves when handling.
- Xylene solution (Wako; cat. no. 244-00086) **! CAUTION** Xylene solution is flammable and a contact hazard. Wear gloves when handling.
- Mounting reagent (O. Kindler, Freiburg; Germany)
- Urea, EP-MB grade (Roche Diagnostics, Mannheim, Germany; cat. no. 11685899001)
- Thiourea (Sigma-Aldrich; cat. no. T7875)
- CHAPS (Wako; cat. no. 345-04724)
- Triton X-100 (GE Healthcare Bio-sciences; cat. no. 17-1315-01)
- DTT (Wako; cat. no. 049-08972)
- Pharmalyte pH 3–10 (GE Healthcare Bio-sciences; cat. no. 17-0456-01)
- CyDye DIGE Fluor saturation dye (GE Healthcare Bio-sciences; cat. no. RPK0283 CY3 and RPK0285 CY5)
- N,N-Dimethylformamide, anhydrous (DMF, Sigma-Aldrich, St. Louis, MO; cat. no. 227056) **! CAUTION** DMF is flammable and a contact hazard. Wear gloves when handling. DMF should be used fresh; i.e., the bottle should not be open for more than 3 months.
- Tris-(2-carboxy-ethyl)phosphine hydrochrolide (TCEP; Sigma-Aldrich; cat. no. C4706)
- Immobiline DryStrip gels 24 cm pI 4–7 (GE Healthcare Bio-sciences; cat. no. 17-6002-46)
- Immobiline DryStrip Cover Fluid (GE Healthcare Bio-sciences; cat. no. 17-1335-01)
- Agarose Prep (GE Healthcare Bio-sciences; cat. no. 80-1130-07)
- Bromophenol Blue (GE Healthcare Bio-sciences; cat. no. 17-1329-01)
- 30% (w/v) acrylamide per 0.8% (w/v) *N,N'*-methylenebis-acrylamide (Wako;

cat. no. 016-15915) ! CAUTION Acrylamide is highly toxic. Wear gloves when handling.
- 1.5 M Tris–HCl buffer pH 8.8 (BioRad, Hercyles, CA; cat. no. 161-0798)
- 87% glycerol (GE Healthcare Bio-sciences; cat. no. 17-1325-01)
- Ammonium persulfate (APS; GE Healthcare Bio-sciences; cat. no. 17-1311-01) ! CAUTION APS is harmful if inhaled or swallowed.
- *N,N,N,N'*-Tetra-methyl-ethylenediamine (TEMED; GE Healthcare Bio-sciences; cat. no. 17-1312-01) ! CAUTION TEMED is harmful if inhaled or swallowed.
- Tris-(hydrocymethyl)aminomethane (Tris–HCl, 5 kg; WAKO; cat. no. 017-16383)
- Gly (10 kg, WAKO; cat. no. 073-00737)
- SDS (Wako; cat. no. 191-07145) ! CAUTION SDS is a respiratory, skin and eye irritant. Wear gloves and mask when handling.
- Bind-Silane (GE Healthcare Bio-sciences; cat. no. 17-1330-01) ! CAUTION Bind-Silane is flammable and a contact hazard. Wear gloves when handling.
- Acetic acid, HPLC grade (Wako; cat. no. 017-00251) ! CAUTION Acetic acid is a contact hazard. Wear gloves when handling.
- Methanol, HPLC grade (Wako; cat. no. 138-06473) ! CAUTION Methanol is a contact hazard. Wear gloves when handling.
- Acetonitrile, HPLC grade (Sigma-Aldrich; cat. no. 27072-7) ! CAUTION Acetonitrile is flammable and a contact hazard. Wear gloves when handling.
- Ammonium bicarbonate (Sigma-Aldrich; cat. no. A6141)
- Trifluoroacetic acid, HPLC grade (TFA; Wako; cat. no. 202-10733) ! CAUTION TFA is flammable and a contact hazard. Wear gloves when handling.
- Sequence grade modified trypsin (Promega, Madison, WI; cat. no. V5111)
- 100% trichloroacetic acid solution (TCA, Wako; cat. no. 200-08005) ! CAUTION TCA is a respiratory system, skin and eye irritant. Wear gloves when handling. ▲ CRITICAL All reagents used in the protocol should be of the highest possible quality.

EQUIPMENT
- CM3050S cryostat (Leica, Wetzlar, Germany)
- Tissue-Tek Cryomold (Sakura Finetechnical, Tokyo, Japan; cat. no. 4557)
- Thermowell sealing tape (Corning Inc., Corning, NY; cat. no. 6569)
- Laser microdissection machine (see EQUIPMENT SETUP)
- MMI membrane slide (a metal frame slide with a membrane for mini CellCut; Molecular Machine & Industries)
- MMI isolation cap (a tube with an adhesive cap for mini CellCut; Molecular Machine & Industries)
- Alumi bath (Asahi Techno Glass, Chiba, Japan; cat. no. CHT-101); a heater with an alumi block
- Immobiline Drystrip Reswelling Tray, for 7–24 cm IPG strips (GE Healthcare Bio-sciences; cat. no. 80-6465-32)
- IEF electrode strips (GE Healthcare Bio-sciences; cat. no. 18-1004-40)
- Filter paper (Chromatography paper 3MM CHR, Whatman, Brentford, Middlesex, UK; cat. no. 3030 909)
- Multiphor II electrophoresis unit (GE Healthcare Bio-sciences; cat. no. 18-1018-06)
- Circulator LTB-250 (AS ONE, Osaka, Japan)
- EPS 3501 XL power supply (GE Healthcare Bio-sciences; cat. no. 18-1130-05)
- Multimeter
- Cell culture dish (100mm × 20mm, Corning Inc.; cat. no. 430167)
- ShakerSRR-2 (AS ONE)
- Equilibration tube set (GE Healthcare Bio-sciences, cat. no. 80-6467-79)
- DALT Gradient Maker with a peristaltic pump (GE Healthcare Bio-sciences; cat. no. 80-6067-65)
- GiantGelCaster (BIO CRAFT, Tokyo)
- Low-fluorescence glass plate (BIO CRAFT)
- Ettan DALT Cassette Rack (GE Healthcare Bio-sciences; cat. no. 80-6467-98)
- Spacers for SE 250 and SE 260 Mini-Vertical Gel Units (10.5 cm × 1.80 mm × 0.75 mm; GE Healthcare Bio-sciences; cat. no. 80-6149-92)
- GiantGelRunner with a dark box (BIO CRAFT); a large vertical electrophoresis apparatus with a cooling system plus a dark box to run the gel in the dark
- Thermo Circulator ZL-100 (TAITEC, Saitama, Japan)
- DarkBox for gel storage (BIO CRAFT)
- Typhoon Trio (GE Healthcare Bio-sciences; cat. no. 63-0055-87).
- KIMTECH Pure CL4 (Kimberly-Clark, Roswell, GA; cat. no. 7605)
- Crew Wipes (Sigma-Aldrich; cat. no. Z23681-0)
- Deep freezer (–80 °C)
- DeCyder 2-D differential analysis software v 4.0, 5.0 or 6.0 (GE Healthcare Bio-sciences)
- Data-mining software developed for DNA microarray data analysis, e.g., Expressionist (GeneData, Basel, Switzerland) and GeneMaths XT (Applied Maths, Sint-Martens-Latem, Belgium)
- Reference markers sheet (GE Healthcare Bio-sciences; cat. no. 18-1143-34)
- Large gel picker PG-100 (AS ONE)
- 96-well thin-wall plate (Asahi Techno Glass)
- FT latex gloves 400 mm (TGK, Tokyo, Japan)
- Ultrasonic cleaner (AS ONE)
- BioShaker MBR-022 (AS ONE)
- AES2010 SpeedVac system (Thermo Electron Corp., Waltham, MA)
- Hitech Tube Crystal (HiTech, Tokyo, Japan; cat. no. M-50001)
- Cell scraper (Corning Inc.; cat. no. 3010)

REAGENT SETUP

Fixative solution for laser microdissection Dilute folmaldehyde with an equal volume of ethanol.

Dehydration solution for laser microdissection Make 70% ethanol (v/v) and 90% (v/v) ethanol in MilliQ water.

10% Triton X-100 Dissolve 10 ml of Triton X-100 in 70 ml of MilliQ water. Make up to 100 ml with MilliQ water. Store at room temperature (~25° C) until use.

Urea lysis buffer 6 M Urea, 2 M thiourea, 3% (w/v) CHAPS, 1% (v/v) Triton X-100. Dissolve 105 g urea, 38.05 g thiourea, 7.5 g CHAPS and 25 ml Triton X-100 in 200 ml MilliQ water. Make up to 250 ml with MilliQ water. Add 3 g Amberlite IRN-150K. Stir for several hours and filter with paper. Aliquot and store at –80 °C until use.

Cy3 and Cy5 dye solution for the analytical gels Centrifuge a tube containing 300 nmol powdered CyDye DIGE Fluor saturation dye at 694*g* for 5 min. Add 60 μl DMF to the tube (final concentration is 5 nmol μl^{-1}). Vortex and centrifuge the tube at 694*g* for 10 s. Store at –20 °C until use.

Cy3 and Cy5 solutions for the preparative gels Centrifuge a tube containing 300 nmol powdered CyDye DIGE Fluor saturation dye at 694*g* for 5 min. Add 20 μl DMF to the tube (final concentration is 15 nmol μl^{-1}). Vortex and centrifuge the tube at 694*g* for 10s. Store at –20 °C until use.

TCEP solution for the analytical gels Dissolve 28 mg TCEP in 50 ml MilliQ water just before use.

TCEP solution for the preparative gels Dissolve 28 mg TCEP in 5 ml MilliQ water just before use.

DTT stock solution Dissolve 1 g DTT in 4 ml MilliQ water. Store at –80 °C until use.

2× urea lysis buffer 6 M Urea, 2 M thiourea, 3% (w/v) CHAPS, 1% (v/v) Triton X-100, 130 mM DTT, 2% (v/v) Pharmalyte 3-10. Mix 900 μl urea lysis buffer, 80 μl DTT stock solution and 20 μl Pharmalyte. Make up to 1,000 μl with MilliQ water.

1 ×x urea lysis buffer 6 M Urea, 2 M thiourea, 3% (w/v) CHAPS, 1% (v/v) Triton X-100, 65 mM DTT, 1% (v/v) Pharmalyte 3–10. Mix 900 μl urea lysis buffer, 40 μl DTT stock solution and 10 μl Pharmalyte. Make up to 1,000 μl with MilliQ water.

Internal control sample Prepare the internal control sample by mixing an equal amount of proteins extracted from laser microdissected tissues from every individual case. Alternatively, a mixture of whole tissue homogenates of the individual cases can be used when contamination from plasma proteins is not severe (you need to confirm this by running 2D-PAGE). Cell-line samples cannot be used as the internal control samples because the *in vivo*-specific spots are not normalized. For a large-scale sample set, prepare an adequate amount of the internal control sample taking into account possible repetition of the experiments.

10% SDS Dissolve 50 g SDS with 300 ml MilliQ water. Make up to 500 ml with MillQ water. Store at room temperature.

Equilibration buffer For 12 IPG gel (24 cm length) mix 90 g urea, 17 ml 1.5 M Tris–HCl (pH 8.8), 87 ml 87% glycerol, 25 ml 10% SDS. Make up to

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250 ml with MilliQ water. Stir the solution for several hours until it reaches room temperature. Add 1.25 g DTT just before use.
Agarose sealing solution Mix 1.0 g agarose prep, 200 ml SDS-PAGE electrode buffer, 200 µl 25 mg ml^{-1} BPB. Heat the solution in a microwave oven. Vortex the solution briefly and prepare 25 ml aliquots of the solution in 50 ml tubes. Leave at room temperature for a short time and tighten the tube cap. Store the agarose gel at 4 °C until use.
10% APS Dissolve 1.7 g APS with 17 ml MilliQ water before use.
Gel buffers for SDS-PAGE See **Table 1** for details of the composition. The light and heavy solutions contain 10% and 15% bis–acrylamide, respectively.
▲ **CRITICAL** Add 10% APS and TEMED just before pouring the solutions into the DALT Gradient Maker. The solution is partially polymerized within 10 min in the GiantGelCaster.
Bind-Silane solution Mix 10 µl Bind-Silane, 200 µl glacial acetic acid, 8 ml ethanol and 1.8 ml MilliQ water.

Table 1 | Composition of gel buffers for SDS-PAGE

	Light solution (1,348.3 ml)	Heavy solution (559.3 ml)
Bis–acrylamide	450 ml	280 ml
1.5 M Tris–HCl pH 8.8	340 ml	140 ml
87% glycerol	0 ml	37 ml
MilliQ water	530 ml	93 ml
10% SDS	14 ml	6 ml
10% APS	14 ml	3 ml
TEMED	300 µl	33 µl

Electrode buffer for SDS-PAGE Make up 35 l for six GiantGelRunner (12 gels). Dissolve 105 g of Tris–HCl and 510 g of glycine in 30 l MilliQ water. Add 350 ml 10% SDS. Make up to 35 l with MilliQ water.
In-gel digestion wash buffer 1 50% methanol. Mix methanol with an equal volume of MilliQ water.
In-gel digestion wash buffer 2 50 mm ammonium bicarbonate. Dissolve 395 mg ammonium bicarbonate in 100 ml MilliQ water.
In-gel digestion wash buffer 3 50 mm ammonium bicarbonate, 50% acetonitrile. Dissolve 395 mg ammonium bicarbonate in 50% acetonitrile.
In-gel digestion dehydration buffer 1 50% acetonitrile .Mix acetonitrile with an equal volume of MilliQ water.
In-gel digestion dehydration buffer 2 100% acetonitrile. Use acetonitrile as is.
Trypsin solution Add 800 µl 50 mM ammonium bicarbonate (Wash buffer 2) into a tube containing 20 µg Sequence Grade Modified Trypsin.
1% TFA Mix 1 ml TFA with 99 ml MilliQ water.
Extraction buffer 45% acetonitrile, 0.1% TFA Mix 1,800 µl 50% acetonitrile (Deydration buffer 1) and 200 µl 1% TFA.
Dissolving buffer 0.1% TFA Mix 50 µl of 1% TFA and 450 µl of MilliQ water.
10% TCA Mix 10 ml of 100% TCA solusion and 90 ml MilliQ water. Store at 4 °C until use.

EQUIPMENT SETUP

Laser microdissection machine There are four types of laser microdissection machines—mini CellCut (Molecular Machine & Industries, http://www.molecular-machines.com), Laser LMD6000 (Leica Microsystems, http://www.leica-microsystems.com), PALM laser microdissection system (PALM Microlaser Technologies, http://www.palm-mikrolaser.com), and a series of laser capture microdissection systems by Arcturus Bio-sciences (http://www.arctur.com), described elsewhere[1]. We use the mini CellCut in this protocol. ▲ **CRITICAL** All four listed laser microdissection systems can perform the basic microdissection tasks of our protocol. The first three dissect large intact tissue areas, whereas the last one recovers individual cells. Contact the suppliers for the details of the machines.

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PROCEDURE

Laser microdissection ● TIMING Day 1, 1 h

1| Embed a fresh surgical specimen in OCT compound in the Tissue-Tek Cryomold. Freeze the OCT-embedded specimen as quickly as possible in a deep freezer (–80 °C) or in dry-ice cold acetone. Seal the frozen sample with an aluminum seal (Thermowell Sealing Tape) to prevent air-drying.
▲ **CRITICAL** Fresh tissue is preferable for laser microdissection. Long-term storage in nitrogen vapor dehydrates the tissues and damages the histological structure. However, because the quality of protein is still suitable for 2D-DIGE, it is worth using such tissues if precise histological observations are not required.
■ **PAUSE POINT** Store the sample at –80 °C until use. If the tissues have not dried excessively, they can be stored for several years.

2| Using a cryostat (Leica C3050S), prepare 4–6 µm thick frozen tissue sections and place on glass microscope slides to confirm the histopathological diagnosis, and 8–12 µm thick sections on membrane-coated glass slides (MMI membrane slide) for 2D-DIGE.
▲ **CRITICAL** Preparation of the frozen tissue sections requires some practice. Consult a pathologist or other suitably trained personnel.
■ **PAUSE POINT** Keep the sectioned tissues in the cryostat at –20 °C until staining. Preferably use the sectioned tissues on the same day.

Hematotoxylin and eosin (HE) staining to confirm the histopathological diagnosis

3| Immerse the 4–6 µm thick frozen tissue sections in fixative solution for 1 min.

4| Wash sections with water for 3 s.

5| Immerse sections in Mayer's hematoxylin solution at 50 °C for 1 min.

6| Wash sections with water for 2 s.

7| Treat sections with water until blue color develops.

8| Immerse sections in 0.5% eosin alcohol solution for 5 s.

9| Wash sections with water until the excess eosin is removed.

10| Dehydrate sections by immersing into 70, 90 and 100% ethanol for 5 s each.

11| Wash sections with xylene solution for 5 s.

12| Add mounting reagent on tissue sections and cover tissues with cover slips.

13| Confirm the presence of target cells by microscopic observation. You can now go on to stain the 8–12 μm thick sections on the MMI membrane slide using the following protocol for 2D-DIGE.

Staining prior to 2D-DIGE

14| Fix sections in 90 % ethanol for 1 min.
▲ **CRITICAL** All solutions should be kept on ice during staining.

15| Immerse sections in Mayer's hematoxyline solution for 1 min

16| Wash sections with water for 3 s.

17| Treat sections with water until blue color develops.

18| Dehydrate sections by immersing into 70, 90 and 100% ethanol for 5 s each.

19| Air-dry sections as quickly as possible at room temperature.
▲ **CRITICAL** Avoiding eosin staining is critical for 2D-DIGE when using the CyDye DIGE Fluor saturation dye[10]. When the target cells are not clearly visible in the hematoxylin-stained sections, use the neighboring HE-stained tissues as a reference. Although protease inhibitors are not included in the staining solutions, we have not observed signs of protein degradation such as poor representation of higher-molecular-weight proteins. We assume that protease activity might be significantly reduced in the first ethanol solution. Ice-cold solutions might also prevent protein degradation.

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20| Dissect an area of the target cells covering at least 3 mm^2 in total, if gels are going to be run in triplicate, using laser microdissection. Recover the cells in 50 μl urea lysis buffer in the MMI isolation cap. Store the sample at −80 °C until use. Refer to the instruction manual for detailed instructions on the operation of the laser microdissection machine, the mini CellCut.
! **CAUTION** All surgical specimens are potentially bioharzardous materials. Use appropriate personal protective attire and dispose of all materials in appropriate containers after the experiments.
▲ **CRITICAL** The total protein concentration of the final sample is too low to be defined by conventional methods such as a Bradford or Lowry assay. Thus, we routinely recover a 3 mm^2 tissue area, extract the proteins and divide the solution into triplicate 2D gels. The average spot intensity between the three gels is then calculated and subjected to further analysis. Sitek *et al.* reported that 1,000 cells were enough to generate a 2D image using our protocol[34]. Although smaller areas could generate 2D images, artificial protein loss during the procedure becomes pronounced when the amount of protein is decreased below a certain level. Although a 3 mm^2 tissue area per three gels empirically generates a 2D image with an adequate number of protein spots, because the exact protein amount can vary between the tissues we recommend using a larger tissue area if the number of the protein spots detected in the 2D image is smaller than expected.

Sample preparation for 2D-DIGE ● TIMING Day 1, 3 h

21| Label the proteins extracted from the laser microdissected cells (option A) and the internal control sample (option B) (see REAGENT SETUP). Perform all procedures in the dark as much as possible.

(A) Protein samples from laser microdissected tissue

(i) Add 4 μl 1.0 M Tris–HCl (pH 8.0), 17 μl urea lysis buffer and 6 μl TCEP solution for analytical gels to a 50 μl protein sample.
(ii) Incubate the mixture at 37 °C for 1 h with quick tapping every 10 min.
▲ **CRITICAL** Brief tapping, not pipetting, during incubation at 37 °C facilitates the protein extraction from the laser microdissected tissues attached to the adherent membrane.
(iii) Add 3 μl Cy5 dye solution for analytical gels.
(iv) Incubate the sample at 37 °C for 30 min with quick tapping every 10 min.

(v) Stop the labeling reaction by adding 80 μl 2× urea lysis buffer.
(vi) Quench the labeling reaction by incubating the sample on ice for 15 min.

(B) Internal control sample (see REAGENT SETUP)
(i) Add 4 μl 1.0 M Tris–HCl (pH 8.0), 20 μl urea lysis buffer and 4 μl TCEP solution for analytical gels to the 50 μl internal control sample containing 15 μg protein.
(ii) Incubate the mixture at 37 °C for 1 h with quick tapping every 10 min.
(iii) Add 2 μl of Cy3 dye solution for analytical gel.
(iv) Incubate the mixture at 37 °C for 30 min with quick tapping every 10 min.
(v) Stop the labeling reaction by adding 80 μl 2× urea lysis buffer.
(vi) Quench the labeling reaction by incubating the sample on ice for 15 min.

22| Mix 160 μl each of the labeled individual and internal control sample. Make up to 1,320 μl with 1× urea lysis buffer.
▲ **CRITICAL** A larger volume of TCEP and dye is used for labeling the individual protein samples than for labeling the internal control sample. The concentration of the protein in the individual samples cannot be measured by any colorimetric method, and excess protein may result in insufficient labeling. Thus we recommend using excess TCEP and dye to label the individual samples. We have not experienced any problems caused by labeling the proteins using excess dye.

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Sample application following in-gel sample application ● TIMING **Day 1–2, 30 min to overnight**
23| Centrifuge the labeled sample at 17,360*g* for 10 min.

24| Spread 430 μl sample evenly in a groove of the Immobiline DryStrip Reswelling Tray. Write sample numbers on the support film of the IPG gel with a waterproof pen. Peel off the protective film from the IPG DryStrip and position the IPG DryStrip on the spread sample gel-side down. Lift and lower the IPG DryStrip gently to coat the entire IPG DryStrip and not to trap air bubbles. Cover the Immobiline DryStrip Reswelling Tray with aluminum foil with wetted paper towel to prevent air-dry and exposure to light. Leave IPG gel in the dark at room temperature overnight[37,38].
▲ **CRITICAL** Although unlabeled proteins cannot be detected in this experiment, the use of forceps and gloves is recommended to avoid protein contamination in the devices, which are later used for protein identification. You can also overlay 1 ml IPG Cover Fluid onto an IPG gel after the sample is fully absorbed by the IPG DryStrip.

Isoelectric focusing ● TIMING **Day 2–3, 1–28 h**
25| Turn on the cooling system (Circulator LTB-250, 20 °C) of the Multiphor II electrophoresis system. Pour a small volume of silicon oil into the electrode tray. Position the rehydrated IPG DryStrip gel-side up in the adjacent grooves of the aligner and place the aligner into the electrode tray respecting anode–cathode orientation. Remove air bubbles between the aligner and the bottom of the electrode tray as far as possible by tilting the tray.

26| Cut two IEF electrode strips to an appropriate length and soak them with 1 ml MilliQ water. Remove excess water completely by blotting the electrode strips with filter paper (Whatman 3MM CHR).

27| Place the IEF electrode strips on each side of the IPG DryStrip so that they have partial contact with the gel. Position the electrode tray on the cooling plate of Miltiphor II unit. Align the electrode bars on the electrode strips.
▲ **CRITICAL** Excess water causes streaking of protein spots. Measure the resistance between the anode (red) and cathode (black) electrode codes of the tray with a multimeter. Infinite resistance value means that there is disconnection at some point.

28| Close the safety lid, connect the electrode lead to the power supply (EPS 3501 XL power supply) and run the isoelectric focusing program as in **Table 2**.
▲ **CRITICAL** Check that there is current immediately after the isoelectric focusing has started. The EPS 3501 XL power supply displays the currency at the μA level.

29| After electrophoresis, place the Immobiline DryStrips in cell culture dishes (100 mm × 20 mm style) with the support film toward the wall. Wrap the dishes with aluminum foil.
■ **PAUSE POINT** The IPG gels can be stored at −80 °C for at least two months.

Table 2 | Ioelectric focusing program.

Step	Voltage (V)	Current (mA)	Wattage (W)	Time (h)	Status
1	500	50	20	1	Gradient
2	1,000	50	20	3	Gradient
3	2,000	50	20	3	Gradient
4	3,500	50	20	3	Gradient
5	3,500	50	20	18	Gradient
6	50	50	20	-h	Step

Casting SDS-PAGE glass plates for second-dimension separation ● TIMING Day 2, 30 min

30| The GiantGelCaster accommodates 13 sets of glass plates for 1 mm thickness gels. Tilt the GiantGelCaster unit back so that it rests on the supporter (**Fig. 2c**, left side). Place the sponge in the groove in the left corner base. Fill the GiantGelCaster unit with 13 pairs of glass plates with separator sheets so that the glass plates can easily be removed from the unit after polymerization. Place the separator sheets to even the level of the stack with the edge of the caster. Place the form gasket in the groove on the faceplate. Position the faceplate and turn the 11 bolts evenly until the form gasket is well compressed. Stand the UltraGelCaster unit horizontally and connect the silicon tube at the bottom (**Fig. 3a**).
▲ **CRITICAL** The GiantGelCaster has a similar structure to the Ettan Dalt II Gel Caster (GE Healthcare Bio-sciences, cat. no. 80-6467-22), but is capable of accomodating larger glass plates. To prevent the GiantGelCaster falling down, put a stopper at the edge of the lab table (**Fig. 3c**). Overtightening the bolts often causes leakage of the gel solution. Wipe the glass plates with ethanol using nonfluorescent paper such as KIMTECH Pure CL4 and Crew Wipes.

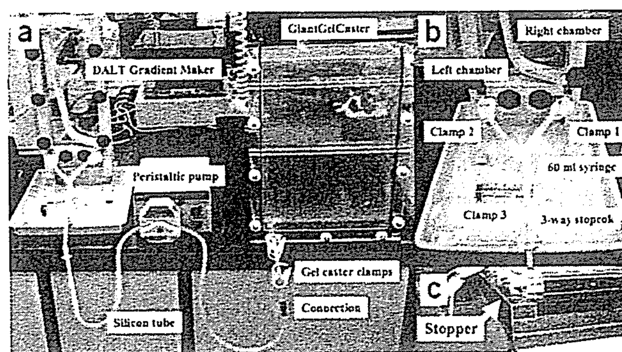

Figure 3 | Appearance of a gradient-gel making system. (a) The DALT Gradient Maker is connected to the Ettan Gradient Maker with a peristaltic pump using a silicon tube. (b) A closer view of the DALT Gradient Maker. A three-way stopcock and a 60 ml syringe are chosen by the authors to remove air inside the tube. Each part is annotated for the better understanding of the protocol. (c) A stopper is fixed on the table, to secure the GiantGelCaster in place.

Making a gradient gel in the GiantGelCaster ● TIMING Day 2, 1 h

31| Connect the gradient maker, the peristaltic pump and the gel caster with a silicon tube with a three-way stopcock and a 60 ml syringe (**Fig. 3a**).

32| Close all pinch clamps (clamps 1–3) and open the three-way stopcock to the syringe (**Fig. 3b**).

33| Gently pour the light solution into the right chamber and the heavy solution into the left chamber of the Ettan Gradient Maker.
▲ **CRITICAL** Make sure that the solutions are at room temperature before use. The amount of APS and TEMED (added in subsequent steps) is adjusted so that the gel looks polymerized within 10 min at room temperature. Just after we take the solutions out of the refrigerator, their temperature is lower than room temperature, and the gel is not polymerized well. It is important to take the solutions out of the refrigerator the evening before making the SDS-PAGE gel and leave them at room temperature overnight. Before using acrylamide solutions, a practice run using water may be needed to understand the process.

34| Open clamps 1 and 3, and flash the silicon tube with light solution using the syringe. Tap the tube to remove air bubbles. Leave 20–30 ml light solution in the syringe. Close clamp 1.

35| Open clamp 2 and flash the silicon tube with the 20–30 ml light solution in the syringe, quickly but gently. Fill the silicon tube with light solution between the 3-way stopcock and the DALT Gradient Maker.

36| Close all clamps, and turn the peristaltic pump on. Adjust the flow rate to 400 ml min^{-1}.

37| Open the three-way stopcock to the peristaltic pump and let the solution go to the GiantGelCaster.

38| Adjust the surface of the light and heavy solution evenly in the DALT Gradient Maker by narrowing the silicon tube with fingers between clamps 1 and 2, and the Y-shaped branch.

39| Stop the pump when the surface of the gel solution reaches about 1 cm below the top of the glass plate.

40| Close the gel caster clamps and disconnect the GiantGelCaster from the DALT Gradient Maker at the connection parts (**Fig. 3a**). Because the pressure from the gel solution inside the GiantGelMaker is very high, double clamps are needed.

41| Wash the DALT Gradient Maker, the silicon tube and the syringes thoroughly with MilliQ water.

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42| Overlay water-saturated butanol onto the top of the gel, and leave the gel at room temperature for 1 h.

43| Overlay the running buffer onto the top of the gel after polymerization, and complete the polymerization overnight at room temperature.
▲ **CRITICAL** Make sure that the solutions are at room temperature before use. The amount of APS and TEMED is adjusted so that the gel looks polymerized within 40 min at room temperature. Just after we take the solutions out of the refrigerator, their temperature is lower than room temperature, and the gel is not polymerized well. We suggest taking the solutions out of the refrigerator the evening before making the SDS-PAGE gel and leaving them at room temperature overnight.

Unloading of the GiantGelCaster ● TIMING **Day 3, 1 h**
44| Tilt the GiantGelCaster unit back so that it rests on the supporter (**Fig. 2c**). Remove the faceplate, the glass plates and separators. Rinse the top surface of each gel between the glass plates with MilliQ water and wash off any acrylamide gel adhering to the glass plates with running water in a sink.

45| Place the glass plates with gels in an Ettan DALT Cassette Rack. Cover the gels with MilliQ water and wrap the top of the glass plate with plastic until use. Discard the unsatisfactory gels, if any, at this stage. Usually, all gels are polymerized well using this protocol.

Equilibration of the IPG strip ● TIMING **Day 3, 30 min**
46| Incubate the IPG strip with 20 ml equilibration buffer shaking gently (ShakeSBR-2) at room temperature for 20 min in the dark.
▲ **CRITICAL** Make sure that the equilibration buffer reaches room temperature before use. Fresh equilibration buffer is always cold because of the urea it contains, and equilibration is not achieved adequately at lower temperature. Prepare the equilibration buffer in the morning, stir it for several hours at room temperature and use it in the afternoon. Leave the IPG strips at room temperature in the dark at least 1 h before equilibration if the strips were stored at –80 °C.

47| Briefly rinse the equilibrated IPG strip with SDS-PAGE running buffer in the equilibration tube at the vertical position. Place the strip on its edge on a filter paper to drain excess moisture.
▲ **CRITICAL** Make sure that the equilibration buffer reaches room temperature before use. Fresh equilibration buffer is always cold because of the urea it contains, and equilibration is not achieved adequately at lower temperature. Prepare the equilibration buffer in the morning, stir it for several hours at room temperature and use it in the afternoon. Leave the IPG strips at room temperature in the dark at least one hour before equilibration, if the strips were stored at –80 °C.

Placing of the equilibrated IPG strip onto the SDS-PAGE gel ● TIMING **Day 3, 30 min**
48| Melt the agarose sealing solution by heating it in the microwave oven.

49| Rinse the surface of the gel with MilliQ water and drain the water completely off with paper.

50| Allow the agarose sealing solution to cool. Add the agarose sealing solution slowly at a constant speed across the entire top surface of the slab gel. Place the IPG strip on top of the gel with the acidic end of the strip z on the left. Push against the support film of the strip using a 0.75 mm thickness spacer (spacers for SE 250 and SE 260 Mini-Vertical Gel Units) until the strip is in contact with the surface of the SDS-PAGE gel. Remove any air bubbles between the surface of the strip and the SDS-PAGE gel.

Set the form gasket in the groove of the GiantGelRunner ● TIMING **Day 3, 30 min**
51| Connect the GiantGelRunner to the external cooling device (Thermo Circulator ZL-100, 15 °C) with silicon tubes.
▲ **CRITICAL** The GiantGelRunner is a larger version of an open SDS-PAGE electrophoresis unit. It can load 27.5 × 40 cm glass plates and comes with a dark box.

52| Pour SDS-PAGE running buffer into the lower tank.

53| Tighten the glass plates to the electrophoresis device with a wedge. Remove the air bubbles at the bottom of the glass plates by tilting the GiantGelRunner unit.

54| Fill the upper tank with SDS-PAGE running buffer.

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55| Connect the electric lead to the power supply (EPS 3501 XL power supply) and start electrophoresis.

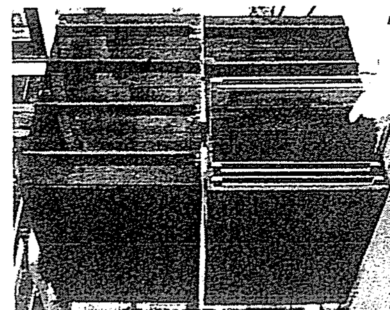

Figure 4 | Dark box used for gel storage. To prevent the fluorescent signal from decreasing, all gels are stored in this box after electrophoresis. The dark box is separated into four chambers and each chamber can contain three sets of glass plates; only three sets of gels are exposed to light when the cover is opened to take a gel out for scanning. The figure shows two dark boxes. This dark box is provided by BIO CRAFT.

Electrophoresis

56| Run the gel at 50 mA constant current per GiantGelRunner (two gels) until the bromophenol blue dye front reaches 1 cm from the bottom of the gel. Cover the GiantGelRunner with a dark box (**Fig. 4**). Second-dimension separation usually takes approximately 13–14 hours.

▲ **CRITICAL** Check for buffer leakage from the GiantGelRunner for the first hour. The voltage usually starts at 180–200 V and ends at 600–650 V per GiantGelRunner (two gels). When the voltage is about half the usual, check for air bubbles under the bottom of the glass plates.

■ **PAUSE POINT** Store the gels in the dark box for several hours until laser scanning.

Image acquisition ● TIMING Day 4, 1.5 h per gel

57| Place the glass plates onto the laser scanner (Typhoon Trio) and scan the gel at the appropriate wavelength according to the manufacturer's instructions.

▲ **CRITICAL** When prescanning, adjust the scanning conditions so that the maximum intensity of the spots is not saturated. We recommend a prescanning resolution of 200 pixels per inch and a final scanning resolution of 100 pixels per inch for a gel area of 24 × 37 cm^2.

58| Import all sets of Cy3 and Cy5 image files of the identical gel into the DeCyder software, as 'gel' files. Detect the spots, selecting the DIA mode of the DeCyder software. A dia file and a corresponding xml file should be created from a pair of Cy3 and Cy5 images.

▲ **CRITICAL** The DeCyder Batch Processor processes multiple gel images under the same conditions. Normalization of a Cy5 image with the Cy3 image on the same gel is achieved automatically. The multiple xml files are then integrated into a bva file. In cancer proteomics, the analysis of hundreds of clinical specimens and three times as many gels when triplicate gels are run is required for the identification of proteomic signatures corresponding to cancer phenotypes. Because a single bva file is not able to include the data of hundreds of gel images, multiple bva files are required to analyze all gel data and be combined later. The different bva files should include identical master gel data so that all identical spots in the different bva files have the same spot number. The following steps demonstrate how to prepare multiple bva files, that will later be integrated.

Image analysis and data mining ● TIMING 60 working days per 130 samples and 390 gels

59| Select a Cy3 image that has a relatively large number of protein spots and better appearance than the other gels. Use this image as the master one, to which all gel images will be matched so that spot numbers correspond to the same spot in every gel. Create a new bva file and import an xml file for the master gel.

60| Add one more xml file into the bva file of the other gels. Select Landmark mode and match at least 400 spots over the entire gel area manually on the additional Cy3 image. The matched spots are recorded on the master gel image.

▲ **CRITICAL** The required number of landmark spots depends on the total number of spots and the quality of gel image. At least 400 landmark spots are required for 5,000 protein spots in a good-quality gel image.

61| Make multiple copies of the bva files including the two xml files. Add 10 more different xml files to each copied bva file. Set the landmarks manually using the recorded 400 spots for all Cy3 gel images.

▲ **CRITICAL** The maximum number of xml files in one bva file is limited depending on the file size of the included xml files. We had the painful experience of having a bva file that contained one hundred 24 × 20 cm^2 gel images broken and never recovered after all landmarking was completed. Although a single bva file may include more than ten xml files of large-format gels, the more gels are included in one bva file, the slower the software works for landmarking. Thus, we suggest having bva files that include ten xml files for a 24 × 37 cm^2 gel.

62| After completing manual landmarking, match the rest of the spots by selecting Process/Match/Match All on the MT mode window menu bar.

63| Export the contents of the bva file to xml files by selecting File/Export Workspace on the menu bar. Repeat this process for all bva files.

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64| Export the spot number and the spot volume from the xml file using the DeCyder XML Tool Box and store them as tab-separated text. Repeat this process for all xml files. Integrate all numerical data into one Excel file. Alternatively, all xml files can be integrated into one file using a function in Expressionist, and then exported as a rel file. Once all data are integrated into a single file, you can use any commercially available data-mining software such as Expressionist and GeneMaths XT to analyze the proteome data obtained by 2D-DIGE[28,29]. We can identify the target protein spots in a preparative gel by image matching, and recover the spots from the preparative gel for protein identification[10].

Preparation of the preparative gel and target spots for in-gel digestion for protein identification ● TIMING 4 d

65| Prepare a protein sample from roughly microdissected tissues, tissue homogenate or tissue cultured cells. Measure the total protein concentration of the sample using conventional methods such as a Bradford or Lowry[39].
▲ **CRITICAL** Make sure that the sample for the preparative gel includes the target spots from the prior image analysis. Spot matching can be perfectly achieved using multiplex 2D imaging of the samples used for the master image and the sample for the preparative gel. Prepare 5 μg of proteins of the sample for the preparative gel and label it with Cy5 CyDye DIGE Fluor saturation dye. Refer to the steps describing the labeling of 15 μg of proteins of the internal control sample. Mix the differently labeled protein samples and apply the mixture on the same 2D gel. Import the image data to the bva file that contains the xml file of the master gel so that all spots are numbered in a coordinated way.

66| Label the protein sample (adjusted to contain 100 μg protein) with CyDye DIGE Fluor saturation dye. Adjust the concentration of protein so that 70.7 μl of sample contains 100 μg/protein.

67| Add 4 μl 1.0 M Tris–HCl (pH 8.0) and 2 μl TCEP solution for preparative gel to the 70.7 μl protein sample.

68| Incubate the mixture at 37 °C for 1 h with quick tapping every 10 min.

69| Add 3.3 μl Cy3 or Cy5 dye solution for preparative gel. Incubate the sample at 37 °C for 30 min with quick tapping every 10 min.

70| Stop the labeling reaction by adding 80 μl of 2× urea lysis buffer.

71| Quench the labeling reaction by incubating the sample on ice for 15 min.
▲ **CRITICAL** In our laboratory, we routinely use the following mass spectrometry setup for protein identification: a Paradigm MS4 high-performance liquid chromatography (HPLC) dual solvent delivery system (Michrom BioResource) for micro-flow HPLC, an HTS PAL auto sampler (CTC Analytics) and a Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron) equipped with a nano-electrospray ionization (NSI) source (AMR). The amount of protein sample can be changed according to the intensity of the target spots. For example, very faint protein spots may need 200–500 μg of protein sample for preparative purposes. However, more protein in the sample does not necessarily increase the protein amount in the spots because not all proteins enter the IPG strip. The degree of separation of the spots from the neighboring ones has to be considered as well to determine the amount of loading protein. Large amounts of protein in the samples often cause poor separation of the neighboring protein spots, resulting in multiple proteins from single protein spots. Thus we are careful not to increase the amount of proteins for the preparative gels. In general, samples with larger protein amounts exhibit problems with isoelectric focusing more frequently, because of the presence of more interfering substances. The sample purification kit (2-D Clean-Up kit, GE Healthcare Bio-Sciences; cat. no. 80-6484-51) is helpful in improving the quality of the protein samples by removing the interfering substances, especially when the whole tissue homogenate is used for preparative purposes.

72| Rehydrate the IPG DryStrip with the labeled sample and perform the first-dimension separation using the Multiphor II.
▲ **CRITICAL** In most cases, the program for isoelectric focusing for the preparative gel can be the same as that for the analytical gel. When a problem is encountered during separation, the longer protocol may be helpful. Although the quality of the sample is a more important factor affecting the resolution of protein spots, it is worth increasing the focusing time first, because it is much easier.

73| Coat the glass plate with Bind–Silane solution so that the gel is immobilized on the glass plate during electrophoresis. Place a small volume of Bind–Silane solution on the entire area of the glass plate and spread the solution using nonfluorescent paper such as KIMTECH Pure CL4 or Crew Wipes in a draft chamber. To allow the excess Bind-Silane to evaporate, leave the coated glass plate for at least 1 h or overnight. Glue two fluorescent reference markers 2 cm apart from the edges of the glass plate at the middle level. Place the coated glass plate into the GiantGelCaster unit, and make a gradient gel immediately as described in Steps 30–45 above.

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74| After equilibration, apply the equilibrated IPG strip onto the second-dimension separation gel, sealing the strip with agarose sealing solution. The procedure for the equilibration and the second-dimension separation is the same as the one for the proteins from the laser-microdissected tissues.
▲ **CRITICAL** Run the preparative gel in a different device than the analytical one. The preparative gel contains a large amount of free fluorescent dye, which migrates into other gels in the same electrophoresis device. This causes severe problems when another gel is run for analytical purposes. Because the analytical gel contains small amounts of proteins, the scanning condition is adjusted to a higher sensitivity than for the preparative gel. The contaminated fluorescent dye is then detected in a large portion of the analytical gel, badly increasing the background of the 2D image.

75| Scan the gel with a laser scanner and submit the acquired image to the DeCyder software. Make a pick list using a function of the software and export it to Large Spot Picker, an automatic spot recovery machine.

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76| Recover the target spots into a 96-well PCR plate with water on the basis of the contents of pick list. Cover the plate with sealing tape (Thermowell Seapling Tape) and store the spots at –20 °C until use. Refer to the manufacturer's instruction manual for the construction of the pick list and the operation of Large Spot Picker.
▲ **CRITICAL** Use gloves and keep every device clean to prevent contamination from proteins. The use of an automated spot-recovering machine can decrease the workload and keratin contamination.
■ **PAUSE POINT** The spots can be stored at –20 °C for up to 4 weeks.

77| Carefully remove and discard the water from the 96-well plate.

78| Add 100 µl of washing buffer 1. Cover the 96-well plate with sealing tape (Thermowell sealing tape). Wash the gel plugs in a sonication water bath with ice for 10 min.

79| Carefully remove and discard the solution.

80| Repeat Steps 78 and 79 four times.

81| Add 100 µl dehydration buffer 1, dehydrate the gel plug for 10 min at room temperature.

82| Carefully remove and discard the solution.

83| Add 100 µl dehydration buffer 2, and dehydrate the gel plug for 10 min at room temperature.

84| Carefully remove and discard the solution.

85| Add 100 µl of wash buffer 2, and rehydrate the gel plug for 10 min at room temperature.

86| Carefully remove and discard the solution.

87| Add 100 µl dehydration buffer 2, and dehydrate the gel plug for 10 min at room temperature.

88| Carefully remove and discard the solution.

89| Add 100 µl wash buffer 2, and rehydrate the gel plug for 10 min at room temperature.

90| Carefully remove and discard the solution.

91| Add 100 µl wash buffer 3, and dehydrate the gel plug for 10 min at room temperature.

92| Carefully remove and discard the solution.

93| Add 100 µl dehydration buffer 2, dehydrate the gel plug for 10 min at room temperature.

94| Carefully remove and discard the solution.

95| Air-dry for 20–60 min at room temperature.

96| Carefully add 5 µl tryspin solution directly to the dried gel plugs, one by one. The gel plug is rehydrated by the incorporation of trypsin solution. A small volume of trypsin solution remains around the gel plug.

97| Cap the 96-well plate tightly and incubate at 37 °C overnight.

98| Centrifuge the plate for 5 s using the AES2010 Speedvac System at the system's fixed gravity (approximately 600*g*; exact speed not crucial), to drive the solution on the wall to the bottom of the well.

99| Add 10 µl extraction buffer directly to the gel plug under visual observation. Extract the peptides in the sonication water bath with ice for 10 min.

100| Carefully transfer the sample to the tube (Hitech Tube Crystal).

101| Add 10 µl extraction buffer directly to each gel plug under visual observation. Extract the peptides in the sonication water bath with ice for 10 min.

102| Completely collect the sample and place all sample segments in the same tube.

103| Reduce the sample volume by evaporation using a vacuum centrifuge (AES2010 SpeedVac System). Watch the volume so that the solution is not completely dried out.
▲ **CRITICAL** The time required for evaporation by the vacuum evaporator depends on the location of the sample in the 96-well plate. Stop the vacuum centrifugation before the most evaporated sample is completely dried.

104| Add 15 µl dissolving buffer.
▲ **CRITICAL** Although the final volume of the samples is not equal between the wells, we add 15 µl 0.1% TFA to all wells when we manipulate a large number of spots such as 96 or 192.
▲ **CRITICAL** Great attention should be paid to avoiding keratin contamination, which can be introduced between electrophoresis and protein digestion. All procedures should be performed in a clean bench in a clean room. The operators should use powder-free gloves that reach to their elbow (FT latex gloves 400 mm). The operators should change the gloves if they touch anything outside the clean bench. Western blotting using an anti-keratin antibody is needed to confirm the positive protein identification when the interpretation of protein identification is difficult due to possible keratin contamination. Careful washing and dehydration remove contaminating materials such as salt and SDS. On the other hand, excessive treatment occasionally creates fine gel particles, resulting in the blockage of the capillary liquid chromatography column. This problem is often encountered when using gel plugs with very sharp edges. Thus, attention should be paid to the shape of the gel plug when a spot picker other than Large Gel Picker is used. We have confirmed that the Ettan Spot Picker (GE Healthcare Bio-sciences, cat. no. 18-1145-28) is compatible with this protocol.
■ **PAUSE POINT** Store the sample at −20 °C until use, for up to 2 weeks.

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● TIMING
The whole procedure takes 5 d for one sample **(Fig. 1).**
Day 1: laser microdissection: 1 h; sample preparation for 2D-DIGE: 3 h; in-gel sample application: 30 min
Day 2: first-dimension separation: 1 h, followed by 28 h of electrophoresis; SDS-PAGE gel preparation: 1.5 h, followed by overnight polymerization
Day 3: second-dimension separation: 2.5 h, followed by overnight electrophoresis
Day 4: image acquisition: 1.5 h; spot picking: 1 h; in-gel digestion: 6 h, followed by overnight treatment
Day 5: in-gel digestion: 3 h

? TROUBLESHOOTING
Unexpectedly poor 2D images can be attributed to many problems during either laser microdissection or 2D-DIGE. Excellent troubleshooting advice can be found in the previous reports[1,5]. However, because multiple problems often contribute to the poor quality of a 2D image, it may be difficult to identify the exact cause of the problem, especially for researchers who are about to start working with 2D-PAGE. There are many variations of 'bad gels', and even if the more experienced scientists could categorize them into small groups based on their experience, one may not find a 2D image similar to theirs in the

troubleshooting guides. In particular, when the sample source is different from yours (e.g., plasma proteins versus cellular proteins), the troubleshooting guides are less helpful because samples with different solubility may present different types of problems. Unfortunately, both laser microdissection and 2D-DIGE are less popular methods, and one may not find an appropriate advisor experienced in both of them.

The first step to finding a solution is to identify the part of the experiment that mainly contributed to the problem, i.e., laser microdissection or 2D-DIGE. For this purpose, we recommend having a good control sample for each experiment. First, according to our experience with 2D-DIGE examining more than 220 malignant tumor cell lines (T.K. and S.H. unpublished observations), the protocol in **Box 1** can surely generate a protein sample that results in a 2D image with the desired quality. Please refer to our recent publications to see a 2D-DIGE image[26,27].

ANTICIPATED RESULTS

The proteome data from tumor tissues are put in context when they are linked to clinico–pathological observations. The proteomic features provided by 2D-DIGE reflect the expression level and posttranslational modifications of proteins. The histological presentation of a tumor provides important information on its biological behavior. Laser microdissection links these two important biological parameters, with the assistance of bioinformatics tools. The proposed protocol will utilize the powerful combination of proteomics and pathology for the benefit of patients.

Using the proposed protocol, one may obtain quantitative information on thousands of protein spots from any type of normal or tumor tissue. Starting with an adequate number of clinical specimens, the data-mining approach allows the identification of proteomic signatures for certain important clinico–pathological parameters, including lymph node metastasis, early recurrence, response to therapy and patients' outcome. The proteins corresponding to the protein spots can be easily determined using in-gel digestion and mass spectrometry. The proteins included in the signature are good candidates for tumor markers for personalized medicine. The results of such proteomic studies will significantly further our understanding of cancer biology.

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BOX 1 | PROTOCOL FOR POSITIVE CONTROL SAMPLE FOR 2D-DIGE

TIMING: 2.5 h

1. Prepare cultured monolayer cells grown in a tissue culture dish with less than 80% confluency. Both normal and cancer cell lines can be used. For suspension cultured cells, please refer to our previous report[41].
2. Wash out culture medium with PBS three times.
3. Pour 20 ml ice-cold 10% TCA solution onto the cells and place the dish on ice as soon as possible.
4. Incubate the cells with ice-cold 10% TCA on ice for 30 min.
5. Collect the cells into a 1.5 ml microcentrifuge tube using a cell scraper.
6. Centrifuge the tube at 694*g* for 5 min.
7. Completely remove and discard the solution, pour ice-cold PBS into the tube and break the pellet by gentle tapping (do not vortex the pellet). This operation should be done within 10 s. Washing with PBS may be critical because the sample pH is occasionally not recovered during later incubation with 50 mM Tris–HCl, pH 8.0, owing to the remaining TCA. The labeling efficiency of CyDye DIGE Fluor saturation dye is dramatically decreased at a pH lower than 8.
8. Centrifuge the tube at 694*g* for 5 min.
9. Remove and discard the solution.
10. Place the tube on ice. Add 500 μl of urea lysis buffer and break the cell pellet as quickly as possible by pipetting.
11. Incubate on ice for 30 min.
12. Centrifuge the tube at 17,360*g* for 30 min.
13. Recover supernatant as a cellular protein fraction.
14. Measure protein concentration with a commercially available kit such as Protein Assay (BioRad, cat. no. 500-0006). Use a 5 μg sample for protein labeling and 2D-PAGE. After adding 1.0 M Tris, pH 8.0, confirm that the pH is approximately 8.0 using a pH indicator.

The use of a smaller electrophoresis device such as the Ettan Dalt*twelve* separation unit may be helpful in finding a solution if you are not very experienced with 2D-PAGE.

Second, if you find that some problems may be due to laser microdissection and protein labeling, prepare the protein sample using a much larger area of laser microdissected tissues. Begin with 5 μg of protein sample for protein labeling and 2D-PAGE. Once the 2D gel is optimized, gradually decrease the area of laser-microdissected tissues used for the protein samples. In our experience, 2D-DIGE can be run using protein samples from an area of laser microdissected tissues smaller than 1 mm^2. However, less protein often results in decreased reproducibility of the experiments, probably due to artificial absorption or loss of the proteins during the experiment procedures. Empirically, protein samples from a 1 mm^2 area of liver cancer, lung cancer, pancreatic cancer or esophageal cancer tissue led to reproducible results. However, the other tissues we did not examine may require a greater amount of tissue for 2D-DIGE. Thus, one should start performing the experiments using at least 1 mm^2 of tissue area first, and once the experiments are going well, consider reducing the area.

Multiple electrophoresis devices and laser scanners are prerequisites for large-scale proteomic studies (**Fig. 2**). The total cost for all equipment is estimated to be equivalent to that of one high-specification LC-MS/MS machine.

In practice, one will find that laser microdissection combined with 2D-DIGE is easier than expected, particularly if one has already mastered running conventional 2D-PAGE. One day of training by the manufacturing company is enough to master both making frozen tissue sections using a cryostat and recovering the tissues using laser microdissection. Once the target cells are clearly detected under microscopic observation, it is a matter of time to complete sample preparation. Protein labeling with CyDye DIGE Fluor saturation dye is simple to perform and almost fool-proof. There is no especially difficult point in running a large-format gradient 2D gel.

The image analysis will take considerable time depending on the number and quality of the gels, and this is the rate-limiting step for proteomic studies using 2D-DIGE. The use of data-mining software has dramatically improved the throughput of numerical data analysis as well as the quality of information obtained from proteome data. More sophisticated image analysis software that will recognize and match the protein spots is desired for even-higher-throughput proteomics.

The proposed in-gel digestion method has been validated on many types of samples and can be used as it is or be further optimized depending on the type of mass spectrometry machine. Even if possible problems are encountered using 2D-PAGE, the quality of the gel images will still be considerably better than with other methods because of the quality of the sample obtained using this protocol; materials interfering with electrophoresis, such as salt and lipids, are removed from the sectioned tissue in the staining process, and samples containing very small amounts of proteins can still be subjected to 2D-PAGE. If the problems encountered are attributable to factors other than sample preparation, the troubleshooting guide will be helpful (see TROUBLESHOOTING).

The number of detected protein spots depends on the type of tissue examined and the way the spots are defined. **Figure 5** shows typical 2D images of protein samples extracted from 1 mm^2 areas of liver cancer tissue and lung adenocarcinoma tissue. These gel images can be used as a reference when one starts the protocol.

The molecular basis for the higher sensitivity of CyDye DIGE Fluor saturation dye compared with CyDye DIGE minimal dye is not fully understood. Saturation labeling is one of the major mechanisms involved, but labeling of additional amino acids may also contribute to the high sensitivity. Indeed, Show *et al.* reported that even a cysteine-negative protein, myoglobin, was labeled with CyDye DIGE Fluor saturation dye[12]. Therefore, it may be possible to further increase the sensitivity of the dye by changing the labeling conditions. CyDye DIGE Fluor saturation dye has potential for functional proteomics. Maeda *et al.* reported that labeling the proteins with CyDye DIGE saturation dye without reducing the reagents allowed the observation of the oxidation status of proteins, leading to the physiological assessment of the proteins[40]. Thus, examining additional possible uses of the dye combined with laser microdissection is a challenging theme to pursue in cancer proteomics.

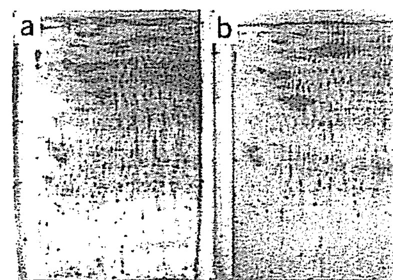

Figure 5 | Typical 2D images of the proteins extracted from a 1 mm^2 area of (**a**) liver cancer tissue and (**b**) lung adenocarcinona tissue. The protein samples were labeled with Cy5 CyDye DIGE Fluor saturation dye and separated using the GiantGelRunner.

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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