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ARTICLE

On-chip *Escherichia coli* culture, purification, and detection of expressed proteins

Moonil Kim · So-Young Lee · Hyunju Choi · Yong-Beom Shin · Sun Ok Jung · Min-Gon Kim · Bong Hyun Chung

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Abstract In a recent study, we reported the results of a rapid high-throughput expression analysis of the affinity-tagged proteins present in total cell lysates, using a surface plasmon resonance (SPR) imaging protein chip system. In this paper, we describe a novel method, which is able to sequentially carry out a recombinant *Escherichia coli* culture, as well as the detection and purification of the expressed proteins on a single microwell chip, fabricated on a two-dimensional thin gold film. Following the induction of the protein on the microwell chip, the *E. coli* cells were lysed on the chip via the addition of lysozymes, and the expressed glutathione *S*-transferase-fused green fluorescent protein (GST–GFP) was then purified on the chip via affinity interaction with the glutathionylated gold surface of the chip. Finally, the expressed protein was directly detected using the surface plasmon resonance (SPR) imaging system. This system saves a substantial amount of time, experimental resources, and labor, by allowing for the complicated and labor-intensive procedures inherent to the production of recombinant proteins to be conducted on a single microwell chip, simply and economically.

M. Kim and S. Y. Lee contributed equally to this work.

M. Kim · S.-Y. Lee · H. Choi · Y.-B. Shin · S. O. Jung · M.-G. Kim · B. H. Chung (✉)
BioNanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology, 115, Yuseong, Daejeon 305–600, South Korea
e-mail: chungbh@kribb.re.kr

Keywords Microwell chip · Surface plasmon resonance (SPR) imaging system · Glutathione *S*-transferase-fused green fluorescent protein (GST–GFP) · On-chip purification

Abbreviations

GST–GFP	Glutathione *S*-transferase-fused green fluorescent protein
IPTG	Isopropyl β-D-thiogalactopyranoside
PDMS	Poly-dimethylsiloxane
SDS–PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPR	Imaging surface plasmon resonance imaging

Introduction

With regard to biological functions including protein–protein and receptor–ligand interactions, as well as small molecule–protein binding, proteins are more important than genes (Templin et al. 2003; MacBeath and Schreiber 2000). Thus far, protein chip technology, among all available techniques, has proven a useful tool for proteomics protocols, including high-throughput screening (HTS), just as DNA chip technology has proven to be a indispensable genomic tool (Phizicky et al. 2003; Albala et al. 2000; Braun and LaBaer 2003). One of the primary factors in proteomics research the ability to generate multiple target proteins in a high-throughput mode. This target protein production must also be achieved in a reasonably

cost-effective manner (Soares et al. 2004; Burbaum and Tobal 2002).

Escherichia coli is commonly utilized as a host in the generation of foreign proteins (Braun et al. 2002). A number of protein expression systems for *E. coli* have previously been developed, which facilitate the purification of proteins, by expressing a target protein with a fusion tag that allows for one-step purification, via affinity chromatography (Nilsson et al. 1997; Sassenfeld 1990). This recombinant fusion protein technology can be applied to a protein array, in which the fusion proteins bind specifically onto the solid surface, modified by an affinity ligand (Tomizaki et al. 2005). Fusion protein arrays have been demonstrated to exhibit good orientation characteristics, as compared to other methods for immobilization (Zhu et al. 2001).

Protein arrays fabricated on thin gold film provide a significant advantage, in that protein-protein interactions can be evaluated using a surface plasmon resonance (SPR) imaging system (Whelan et al. 2002; Smith and Corn 2003; Steiner 2004). Thus far, most of the currently available protein arrays are predicated on methods of detection employing enzymatic or fluorescent tags (Phelan and Nock 2003; Kawahashi et al. 2003). By way of contrast, SPR imaging is a label-free, surface-sensitive spectroscopic technique, which is capable of detecting multiple biomolecular interactions occurring on two-dimensional thin gold film, via the measurement of changes in the local index of refraction upon adsorption (Fivash et al. 1998). Currently, SPR imaging systems have been used in the rapid detection of recombinant proteins expressed in *E. coli* (Jung et al. 2004; Jung et al. 2005). However, the expression and purification of recombinant proteins in *E. coli* remains a complicated task, requiring a great deal of manual skill.

In this study, we have developed a microwell chip system in order to simplify the conventional cell culture protocol. This system simplifies the procedures inherent to protein expression, detection, and purification, all of which previously necessitated expensive devices, abundant experimental resources, and intensive labor. In this novel system, the principal steps in the generation of recombinant proteins, including cell culture, IPTG induction, cell lysis, and the purification and detection of the recombinant protein, are integrated onto a single microwell chip. This, then, results in a significant reduction with regard to the time, experimental resources, and labor associated with protein preparation.

Materials and methods

Well structures on slide glass (22×22 mm^2) were achieved quite simply, using a PDMS (poly-dimethylsiloxane) elastomer. A flat plastic dish was filled with PDMS, and all bubbles were eliminated. After the PDMS was cured for 3 h at 60°C, a 2 mm thick PDMS plate was stripped from the plastic dish. Holes 2 mm in diameter were then made in the PDMS plate, using a hole punch. In order to permanently bond the PDMS plate to the glass slide, the surface of the punched PDMS plate was oxidized using O_2 plasma. The plasma-treated PDMS plate was placed onto, and then pressed down upon, a slide glass, which had been precleaned with piranha solution [70% (v/v) H_2SO_4, 30% (v/v) H_2O_2].

The procedure by which the gold-surfaced microwell chip used for SPR detection was fabricated was similar to that of the glass-surfaced well chips used in the initial tests of the on-chip cell culture and induction protocols. First, the bottom substrates for SPR measurement were prepared with a commercial electron-beam evaporator, in order to deposit a thin gold film (47 nm) with an adhesion-enhancing chromium underlayer (2 nm) onto the clear slide glasses. The punched PDMS plates were then fabricated and treated with O_2 plasma, using the method described above. A solution of 3-mercapto-propyl trimethoxy silane (1%) was applied to the plasma-treated surface of the PDMS plate, then dried with N_2 gas. The PDMS plate was then positioned on the gold substrate. A subsequent baking step, for 1 h at 120°C, was required to generate adequate adhesion between the PDMS and the slide. After baking, the PDMS plate was permanently attached to the gold surface.

The microwell chips were immersed for 16 h in ethanol containing 10 mM 11-mercapto-1-undecanoic acid (MUA). The self-assembled monolayer (SAM) of MUA on the gold surface of the microwell chip was activated as a hydroxysuccinimidyl ester, via 10 min of incubation with a mixture of 0.1 M *N*-hydroxysuccinimide (NHS) and 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in distilled water. After surface activation, a PBS buffer (pH 7.4) containing amino-dextran (1 mg/ml) was applied to the gold surface of the microwell chip, and allowed to react for 1 h. The dextran surface, which is hydrophilic, was then activated for 2 h using 0.1 mg/ml *N*-(*β*-maleimidopropyloxy) succinimide ester (BMPS) in PBS buffer (pH 7.4). The chips were then incubated for 2 h in PBS buffer (pH 7.4) containing 1 mg/ml L-glutathione (reduced form), in order to modify the activated gold surface. The chips

were subsequently washed with distilled water. The resultant glutathionylated microwell chips were stored in PBS buffer (pH 7.4) at 4°C. The MUA and NHS were purchased from Sigma-Aldrich. The EDC and BMPS were purchased from Pierce (IL, USA). The amino-dextran (M.W. 500,000) was purchased from Molecular Probes (OR, USA).

For the cloning of the GST–GFP fusion gene, the entire gene (*Aequorea Victoria* GFP, GenBank accession number X83959) encoding for green fluorescent protein (GFP) was amplified with the 5′ (GAT GGA TCC AGT AAA GGA GAA GAA), and the 3′ (GAT TCT AGA TTA TTT GTA TAG TTC), via the polymerase chain reaction (PCR). The 5′and 3′ termini were designed to harbor *Bam*HI/*Xba*I restriction enzyme cleavage sites. The PCR products were purified with a DNA purification kit (Qiagen), and digested with the restriction enzyme *Bam*HI/*Xba*I sites. The resultant DNA fragments were then ligated with a pGEX-KG vector (Guan and Dixon 1991) using a ligation kit (Takara, Japan) (pGST–GFP). The transformed cells, *E coli* BL21(DE3)/pGST and *E. coli* BL21(DE3)/pGST–GFP, were cultured in 5 ml of Luria-Bertani (LB) media and allowed to grow at 37°C to OD 0.6 prior to the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). After the administration of IPTG, the cells were cultured for an additional 4 h, after which the sample solution (10 μl) was mixed with 10 μl of the sample buffer [2× containing 125 mM Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 1 μg/ml of bromophenol blue (BPB)]. This mixture was then boiled for 3 min, and loaded into a polyacrylamide gel well. The proteins were separated via 10% SDS-polyacrylamide gel electrophoresis, and visualized via Coomassie's brilliant blue R-250 staining.

Five μl of *E. coli* suspension harboring the pGST–GFP plasmid (BL21/pGST–GFP) in LB media were incubated for 6 h on a microwell chip at 37°C prior to the addition of 1 mM IPTG. The cells were then treated with IPTG, cultured for an additional 3 h without agitation, and lysed with lysozymes (50 units/well). Following the lysis of the *E. coli* cells on the microwell chip, the cell lysates were extracted. The gold surfaces of the microwell chips were washed 3× with distilled water, followed by the addition of 5 μl of 30 mM GSH in order to purify the GST–GFP fusion protein from the glutathionylated gold surfaces.

In order to acquire SPR images of the GST–GFP recombinant protein on the gold surfaces of the microwell chips, we used a two-dimensional surface plasmon resonance (2D-SPR) imaging system. A 150 W quartz tungsten-halogen lamp (Schott, Germany) was employed as a light source, and the light was delivered to the goniometer arm (Physik Instrumente, Germany) using a liquid light guide (Oriel Instruments, USA). The light was collimated by the lenses, and passed through a narrow interference filter (750 nm, $\Delta\lambda$ = 2 nm; Oriel Instruments, USA) and a polarizer (Newport, USA), in order to convert the monochromatic and linear polarized beams, respectively. The microwell chip on which the recombinant proteins had been captured was optically coupled with a prism coupler (Korea Electro-optics, South Korea) using index matching oil (n_D = 1.517), and positioned in the center of the goniometer. The images reflected from the microwell chips were then photographed with a 1/2 inch charge coupled device (CCD) camera (Sony, Japan), while the contrast images were monitored on a personal computer. A combination of lenses was positioned in front of the bare CCD chips, in order to obtain clear images. These images were then stored digitally in a personal computer, using a B/W frame grabber (National Instrument, USA).

For fluorescence detection, 2 μl of the elution fractions from the cell lysates, either with or without IPTG treatment, were transferred onto slide glasses. Fluorescence images of the GST–GFP recombinant proteins were then obtained using a GenePix 4200 A 488 nm laser (Axon Inc., USA).

Results and discussion

Conventional SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting techniques have been used extensively in the analysis of target proteins. The primary drawback of these procedures is that they are notoriously laborious and time-intensive. We demonstrated previously that the specific binding of GST fusion proteins onto glutathionylated gold chips can be detected using the SPR imaging system, thereby facilitating the rapid detection of recombinant protein expression in a high-throughput manner, without the necessity of conducting traditional laborious procedures such as SDS–PAGE and Western blotting (Jung et al. 2004). However, in spite of the rapid and simultaneous detection of multiple recombinant proteins expressed in *E. coli* with affinity tags to facilitate purification, this method still requires some fairly labor-intensive steps, most notably the procedures associated with cell culture (Jung et al. 2004). Recently, several research groups have developed on-chip cell culture systems for monitoring allergic responses (Matsubara et al. 2004), for the analysis of isolated single cells under contamination-free conditions (Inoue

et al. 2005), and for monitoring the adaptation process of bacterial cells during changes in nutrient concentrations (Umehara et al. 2003). Although on-chip cell culture systems integrate cell culture, chemical treatment, and detection steps (McClain et al. 2003; Wang et al. 2001), these systems require a fairly elaborate set-up, and several additional devices or apparatus. These requirements remain a hindrance to the routine use of on-chip cell culture systems in proteomic analyses and disease diagnostics. In this study, we attempted to devise an on-chip micro-cell culture system that integrates cell culture and recombinant protein purification steps on a single chip, with no more need for such peripheral devices. In service of this goal, the fabrication of an on-chip micro-cell culture system constitutes an unavoidable first step. Poly-dimethylsiloxane (PDMS), which is known to be nontoxic when combined with biological materials in liquid solutions, exhibits hydrophobicity, and carries no charge. Many previous studies have reported the development of microdevices that utilize PDMS, as this substance exhibits high-fidelity production when cast in a mold with structures on the sub-micron-scale (Beebe et al. 2000; McDonald et al. 2000). Considering these advantages of PDMS in the context of the development of an on-chip micro-cell culture system, we fabricated a PDMS-based microwell chip, and constructed wells in order to facilitate the cell culture procedure (Fig. 1). Moreover, to permanently attach the PDMS plate to the gold plate, we treated the surface of the punched PDMS plate with 3-mercapto-propyl trimethoxy silane (3-MPTS), in order to foster the formation of thiol-groups (-SH; Fig. 1). These microwell chips were comprised of four cell culture wells, each of which were 2 mm in diameter, and could accommodate 5 ml of cell suspension. Using the microwell chip fabricated in this study, different four proteins from *E. coli* can be expressed and purified. Moreover, as many as 36 recombinant proteins can be immobilized on the same chip (2.2×2.2 cm^2) by reducing both the diameters of the wells and their spacing. Our method provides a basis for the fabrication of microwell chips that can be adopted for cell cultures, allowing for the on-chip purification of recombinant proteins.

The conventional procedures used for on-chip protein studies, such as the analysis of protein-protein interactions, involve labor-intensive experimental steps, large-scale production protocols, and the purification of target proteins, all of which must be completed prior to the immobilization of purified proteins on the chip. Thus, in order to simplify these time- and resource-intensive procedures, we attempted to devise a method for the on-chip micro-cell culturing and purification of recombinant proteins. First, in order to determine whether the recombinant proteins in *E. coli* could be induced in a micro-scale cell culture on a chip, we fabricated well chips with glass surfaces (glasswell chips). The volume of each of the wells on these glasswell chips was 5 μl (Fig. 2a). The pGST–GFP plasmid was then cloned for the GST–GFP fusion gene and was

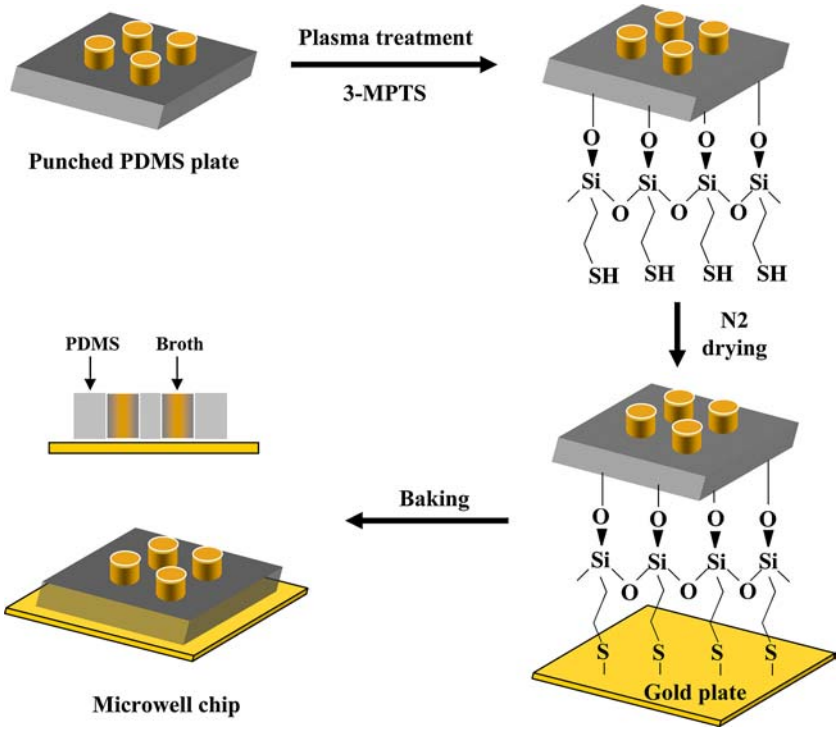

Fig. 1 Schematic diagram of the microwell chip. A microwell chip designed for an on-chip micro-cell culture system. The microwell chips were composed of four wells for cell culture, each 2 mm in diameter, and with a cell suspension capacity of 5 μl

transformed into *E. coli*, and the bacterial cells harboring the pGST–GFP plasmid were incubated on a glasswell chip and allowed to grow to an OD_{600} of 0.6 prior to the addition of IPTG for the expression and purification of the *E. coli* recombinant proteins on the glasswell chips. The cells were treated with 1 mM IPTG, incubated for an additional 3 h, and lysed using lysozyme (50 units/well) only on the glass chip, with no detergent, as detergents might have hindered the glutathione surface modification. The 5 μl of total cell lysates incubated in each of the wells on the glasswell chips were then electrophoresed on 10% SDS–PAGE, and visualized via Coomassie's brilliant blue staining. As is shown in Fig. 2a, the expression of GST–GFP in the micro-scale cell cultures was detected on the SDS–PAGE, indicating that the recombinant proteins, even at a volume of only a few μl, could be induced on the chip. On the basis of this result, the transformed *E. coli* cells were evaluated with regard to the on-chip expression and purification of recombinant proteins on gold-surfaced microwell chips, using the same procedures as were followed with the glasswell chip. After the *E. coli* cells in the wells had been lysed with lysozymes, the BL21/pGST–GFP cells were incubated for an additional 1 h, in order to evaluate specific binding between GST and GSH occurring on the microwell chip. Next, 5 μl of *E. coli* cell lysates were separated on SDS–PAGE, and visualized via Coomassie's brilliant blue staining. Unlike our observations with the glasswell chips, we detected no GST–GFP in the cell lysates on the microwell chips (Fig. 2b). These results verified that the GST–GFP recombinant protein had bound tightly to the GSH-immobilized gold surface, as GSH allowed for the specific immobilization of GST-fused proteins on the microwell chips. On the basis of this result, we surmised that the purification of GST–GFP recombinant proteins could, indeed, be achieved on microwell chips, due to the specificity of the GSH-immobilized surface for the GST-fused proteins.

After the cell lysates remaining in the wells on the microwell chip had been removed, the gold surface of the microwell chip was washed three times in distilled water, and the GST–GFP fusion proteins were analyzed directly, using the SPR imaging system, in order to evaluate the amount of recombinant proteins expressed. These SPR images were captured at a slightly smaller incident angle than the SPR angle of the well surface corresponding to the non-induced cell culture, in which the difference of the intensity between both well images was at its maximum (Fig. 3a). Therefore, the brighter spots indicate the affinity binding of the target proteins to the glutathionylated microwell chips. As is shown in Fig. 3b, the brighter SPR images, as compared to those obtained with the IPTG-non-induced cell suspension, were clearly observed in the IPTG-induced cell culture, whereas visible images were seldom obtained with non-induced cell cultures. This supports the notion that our SPR imaging analysis

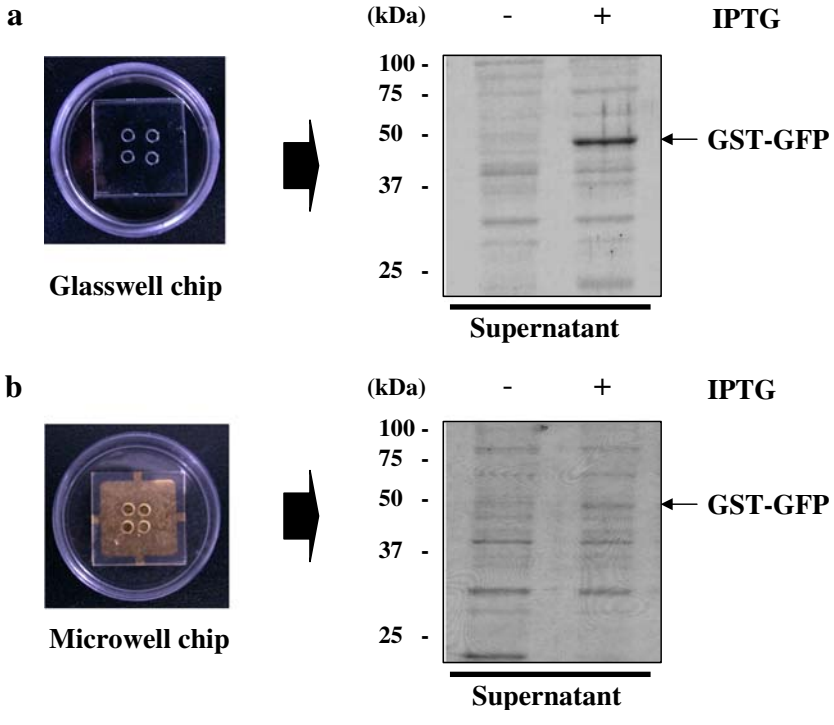

Fig. 2 Microwell chip and SDS–PAGE analyses of the expressed proteins. **a** Coomassie-stained SDS–PAGE from *E. coli* cell lysates on a glasswell chip for the on-chip cell culture and recombinant protein induction tests. **b** Microwell chip for SPR imaging analysis and on-chip purification

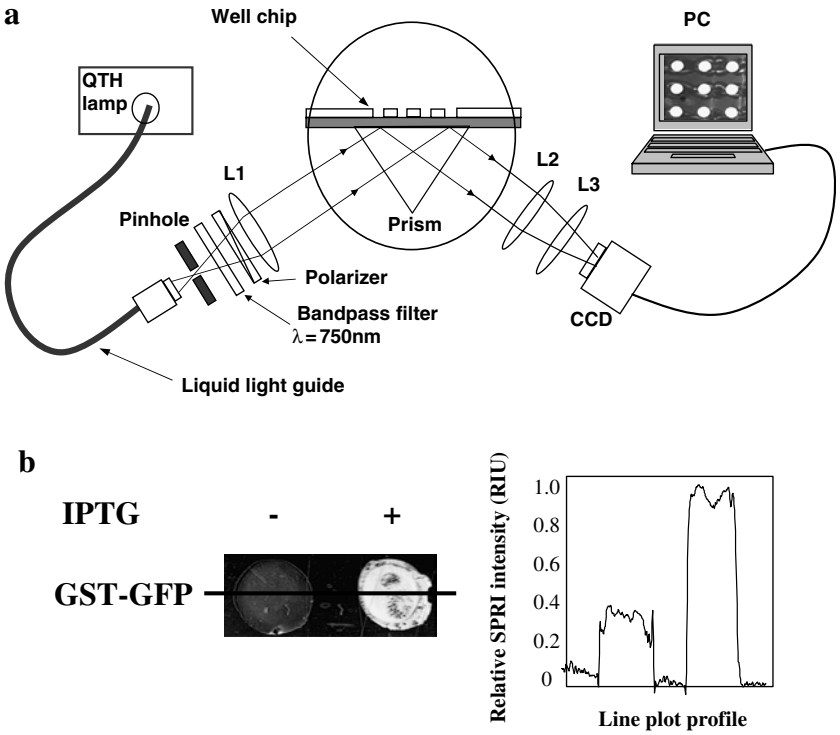

Fig. 3 SPR imaging analysis. **a** SPR imaging system. A p-polarized and monochromatic light beam was directly focused on a prism coupler (FD110, $n \sim 1.76$; Korea Electro-optics, Korea). The image reflected from the *gold* surface of the microwell chip was photographed with a 1/2 in. monochromatic CCD camera (Sony, Japan). A combination of lenses (L2, L3) was positioned in front of the bare CCD in order to acquire a clear image. This image was then digitally stored in a personal computer using a B/W frame grabber (National Instrument, USA), and analyzed further using SPR imaging software (K-MAC Co., Korea). **b** An SPR image showing the binding of the GST–GFP onto the glutathionylated *gold* surface on a microwell chip. The *line profile* taken across the image shows the specific binding of the GST–GFP to the GSH-immobilized gold surface

corresponds to the SDS–PAGE analysis shown in Fig. 2b. In addition, on the basis of SPR imaging results, the reproducibility of this recombinant proteins were around 95%, indicating that there is low well-to-well variability (data not shown). These findings, then, suggest strongly that the GST–GFP recombinant proteins from *E. coli* can be successfully induced in a micro-scale cell culture, and can be detected on the microwell chips with an SPR imaging system.

In order to purify the GST–GFP fusion protein from the glutathionylated gold surface, 5 μl of 30 mM GSH were added to the wells on the microwell chip immediately after the acquisition of the SPR imaging measurements of GST–GFP, for 1 h at room temperature, and subsequently resuspended three times by pipetting. Figure 4a shows the levels of purified GST–GFP recombinant protein directly eluted from the glutathionylated gold surface of the microwell chip. When the amounts of GST–GFP recombinant proteins in the gold surface fraction and the elution fraction from the IPTG-induced *E. coli* cell lysates were analyzed via SDS–PAGE, the single band of 46 kDa proteins containing the GST (26 kDa) (GST–GFP) protein was detected only in the elution fraction, whereas the gold surface fraction evidenced no detectable band for the GST–GFP fusion protein (Fig. 4a). The fact that the GST–GFP was detected only minimally in the gold surface fraction, but prominently in the elution fraction, indicates successful on-chip protein purification. The on-chip purification results are shown in Table 1, and represent the average yield from four preparations. The final purity and recovery yields in the elution fraction were 84 and 17, respectively, but were 12 and 2%, respectively, in the suface fraction. This indicates that the GST–GFP had been purified effectively on the microwell chip. In addition, when analyzed by laser-scanning densitometric SDS–PAGE, the degree to which the recombinant GST–GFP protein had been expressed and purified on the microwell chip was comparable to the results gleaned using GST affinity chromatography, the conventional recombinant protein expression and purification technique (data not shown).

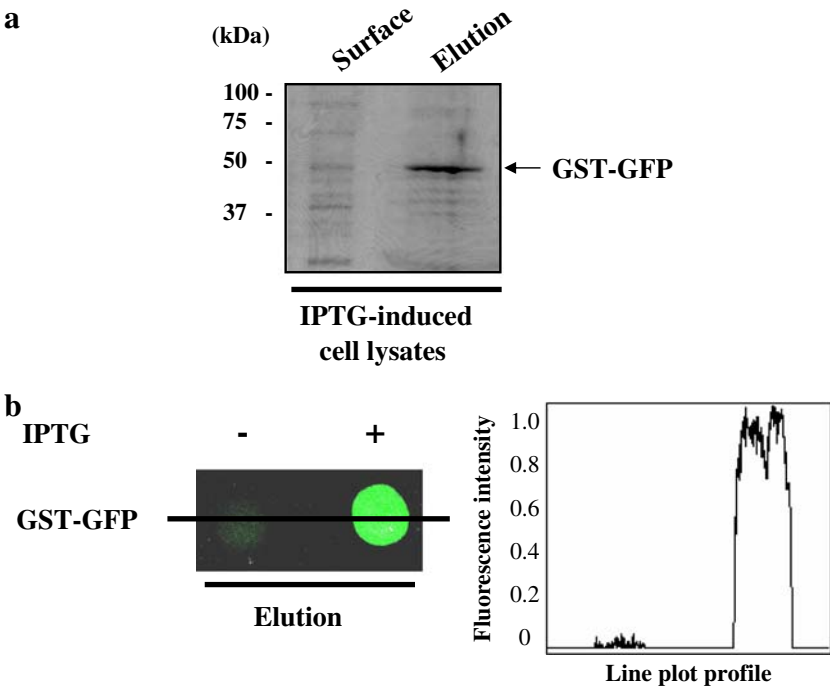

Fig. 4 Purification of GST–GFP recombinant proteins in *E. coli* on a microwell chip. **a** Coomassie-stained SDS–PAGE from the IPTG-induced cell lysates in the glutathionylated gold surface fraction, and the elution fraction treated with GSH after the addition of IPTG. **b** Fluorescence image of the GST–GFP recombinant protein, acquired by a GenePix 4200 A 488 nm laser (Axon Inc., USA). Fluorescence images of the GST–GFP from the elution fractions from the IPTG non-induced and induced cell lysates were quantified using the GenePix Pro 6.0 system (Axon Inc., USA)

Table 1 On-chip purification of recombinant GST–GFP expressed in *E. coli*

	Tatal protein (μg/ml)	GST–GFP (μg/ml)	Purity (%)	Recovery (%)
Total extract	2.61	1.17	45	100
Elution fraction	0.25	0.21	84	17
Surface fraction	0.24	0.03	12	2

We also conducted a fluorescence detection trial on the GST–GFP recombinant proteins, in order to determine whether or not the GST–GFP was active. Two microlitres of the elution fractions from the cell lysates, either with or without IPTG treatment, were transferred onto slide glasses. Fluorescence images of the the GST–GFP recombinant proteins were then obtained using a GenePix 4200 A 488 nm laser (Axon Inc., USA). As is shown in Fig. 4b, the elution fraction obtained from the IPTG-induced *E. coli* cell lysates exhibited prominent fluorescence in the images acquired by the GenePix 4200 A laser scanner, whereas the cell lysates to which IPTG had not been administered evidenced no detectable fluorescence. These results suggest that the GST–GFP recombinant protein had retained its functional stability after the on-chip purification.

In conclusion, we successfully expressed and purified the GST–GFP fusion protein in *E. coli* on a microwell chip, even in the absence of a shaking incubation step. Our data showed that the active GST–GFP recombinant protein present in the total cell lysates had been effectively concentrated and purified on the microwell chip without comparable nonspecific binding protein in *E. coli*, by virtue of the specificity of affinity between GST and GSH. Although our system requires a robust microwell chip, this has distinct advantage that the cell cultivation, protein expression detection, and purification steps, normally separate, can now be integrated on a single microwell chip. In this study, we used GST-tagged protein as a target protein, and a glutathionylated gold surface on the microwell chip. This system should be applicable to a host of other fusion protein assays. For example, a maltose-coated gold surface could be used to study MBP-tagged proteins, and a Ni^{2+}-coated gold surface for His-tagged proteins. Collectively, our results in this study indicated that on-chip micro-cell culture systems are rapid and simple systems, and can be used to verify the expression of fusion proteins, as well as to purify the recombinant proteins captured on the microwell chip. This system may prove particularly useful in the development of drugs that require the high-throughput expression and purification of recombinant proteins, with a significant reduction in time and labor as compared to more traditional methods.

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