In situ phage screening; a method for identification of subnanogram tissue components in situ *

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Running title: Nanoscale protein identification in situ
Abstract

We have established a novel method, in situ phage screening (1ISPS), to identify proteins in tissue microstructures. The method is based on the selection of repertoires of phage-displayed antibody fragments with small samples of tissues micro-dissected using a laser. Using a human muscle frozen-section with an area of 4,800 µm² as a model target, we successfully selected monoclonal antibody fragments directed against three major (myosin heavy chain, actin and tropomyosin-α) and one minor (α-actinin 2) muscle constituent proteins. These proteins were present in the sample in amounts less than one nanogram, and the antibodies were used to visualize the proteins in situ. This shows that the use of ISPS can obtain monoclonal antibodies for histochemical and biochemical purposes against minute amounts of proteins from microstructures with no requirement for large amounts of samples or biochemical efforts.
Introduction

In the post-genomic era, nano-scale technologies such as femtomole-ranged mass spectrometry (1) have become indispensable for proteomic analyses to clarify changes in protein components and abnormality of protein functions in human diseases. Here, we describe a novel nano-scale method, in situ phage screening (ISPS), which enables us to identify protein components in microstructures seen under microscopy. The method involves the combination of phage antibody display technology (2-5) with laser micro-dissection technology (6, 7). For these purposes, we used a library of single chain variable fragments (scFv) of antibodies (2, 3) fused to gene III of M13 phage and expressed at the tip of the phage particles (3, 8). For microdissection of tissues, we developed a new laser-microdissector by combining an industrial-use laser cutter and an inverted microscope. The procedure involves isolation of the target microstructures from the surrounding tissues on glass by dissection, and then incubation with the phage antibody library. Phage antibodies were isolated after a single round of selection, and their specificities were identified by immunohistochemistry and Western blotting. Target antigens were identified by immunoscreening of cDNA expression libraries using the monoclonal phage antibodies.

The clinical targets of ISPS are those characterized by abnormal or yet unknown protein accumulations which closely relate with pathogenetic mechanisms, such as subcutaneous deposits or inclusion bodies and extracellular plaques often seen in neuromuscular and other degenerative diseases. ISPS is highly advantageous when the target microstructures can hardly be collected by standard biochemical methodologies; for instance, targets are
very small (up to 1 μm in size) or rare (seen in few cells), or they appear in complex pathophysiological structures.

Since muscle cells consist of relatively small numbers of proteins, we employed human skeletal muscle as a model target of ISPS. In this paper, we describe model experiments of ISPS on human muscle, where we successfully identified the three major and one minor muscle antigens from an area of 4,800 μm² microfragments.

**Experimental Procedures**

**scFv phage library and production of recombinant phages.** A human synthetic scFv library Griffin. 1 (9) was provided from Dr. G. Winter (MRC, UK) and amplified in our laboratory. In this library, the V\textsubscript{H} and V\textsubscript{L} sequence connected with a spacer sequence (3, 8) was inserted into an Nco I - Not I restriction site and followed by the M13 gene III sequence in phagemid vector pHEN2. Recombinant scFv phages were rescued in *Escherichia coli* suppressor strain TG1 in the presence of helper phage VCSM13 (Stratagene Cloning System, USA) as described previously (9, 10). Briefly, bacteria carrying scFv phagemid were cultured in a 2 x YT medium (11) containing 100 μg/ml ampicillin and 1% glucose and infected in the log phase (absorbance at 600 nm [A\textsubscript{600}] was 0.5) with VCSM13 at a ratio of 1:20 (number of bacterial cells/phage particles) for 30 min at 37°C without shaking. The bacteria were pelleted by centrifugation, resuspended in a 2 x YT medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin (4 volumes of the original), and grown overnight at 30°C. Phages were purified from the culture supernatant by polyethylene glycol precipitation (2) and resuspended in TBS (20 mM Tris-Cl pH 7.6, 0.15 M NaCl). The working solution of the phage library was prepared at
the concentration of $10^{13}$ colony forming units (cfu)/ml. An anti-thyroglobulin scFv phage was also provided by Dr. G. Winter.

**Selection of scFv phages in situ.** Histologically normal biopsy specimens of skeletal muscle (biceps brachii) were provided by the NCNP research resource network (Kodaira, Tokyo) after obtaining the informed consent of the patients. Transverse (experiments A and B) and longitudinal (experiments C to E) frozen sections that were 2 $\mu$m thick were cut with a cryostat, attached to silanized slides (Dako), and subjected to laser micro-dissection. Spaces 15 $\mu$m wide surrounding 40 x 40 $\mu$m$^2$ square microfragments were made with pulsed shots of the ultraviolet laser. After the sections were immersed in acetone for 5 min and air-dried, they were blocked with TBS-10% (weight/volume) skim milk for 2 h and then incubated with the phage library ($5 \times 10^{11}$ cfu in 50 $\mu$l TBS in experiments A and B or in 75 $\mu$l TBS-10% skim milk in experiments C to E) for 12 h at room temperature. After washing with TBS-0.05% Tween 20 (TBS-T) four times x 30 min, the sections were overlaid with 0.2 ml of TBS-T. Three microfragments of 40 x 40 $\mu$m$^2$ were then collected into a microfuge tube with a hand-made micropipette (inner diameter: 60-80 $\mu$m) together with a minimal amount of overlaid TBS-T, and the microfragments were washed three times by brief centrifugation with 0.2 ml TBS-T each. After washing, the contents of the tube were examined microscopically to determine whether the three microfragments remained in the tube.

In the direct colony recovery protocol, phages were eluted from the pelleted fragments with 100 $\mu$l of 1.4% triethylamine for 10 min at room temperature with vigorous shaking followed by neutralization with 50 $\mu$l of 1 M Tris-Cl pH 7.4. A TG1 culture (1 ml) in the log
phase ($A_{600} = 0.5$) was infected with the eluted phage solution for 30 min at 37 °C and plated on LB-agar (11) containing 100 µg/ml ampicillin and 1% glucose. All of the ampicillin-resistant colonies obtained, which were carrying scFv phagemid, were subjected to the analysis of specificity.

In the PCR-fragment subcloning protocol, the three pelleted microfragments were suspended in 10 µl of water, heated at 95° C for 5 min, and then rapidly cooled in ice. The Fv sequences derived from the phages bound to the microfragments were amplified by PCR using forward (5’-CGGATAACAATTTCACACAGGAAAC-3’) and reverse (5’-CTATGCGGCCCCCATTCAGATC-3’) primers. The reaction (50 µl) contained 5 µl of a 10-fold-concentrated Taq reaction buffer (Takara, Japan), 0.8 µM each of the forward and reverse primers, 0.2 mM dNTPs (Takara, Japan), the heat-treated sample (10 µl as described above), and 2.5 units of Taq polymerase (Takara, Japan) and was kept in ice. After heating at 98° C for 1 min, 38 cycles of PCR (60° C for 20 sec, 72° C for 1 min, and 95° C for 20 sec) were performed and followed by incubation at 72° C for 3 min. The PCR product (0.95 kbp) was digested with Nco I and Not I, ligated with Nco I- and Not I-digested pHEN2, and transformed into TG1. The bacteria were plated to form ampicillin-resistant colonies. It was confirmed that no PCR products were obtained by using the final wash-supernatant of the muscle microfragment (Fig. 1). A portion of the colonies was subjected to analysis of scFv specificity.

**Specificity of scFv phages.** The Fv sequence in each colony was amplified by PCR (30 cycles of 95° C for 15 sec, 60° C for 15 sec, and 72° C for 1 min) using the same primer set as described above, digested with Hae III and electrophoresed on 4.5% agarose for
fingerprinting. The monoclonal phage was rescued from the representative clones showing unique patterns on fingerprinting and analyzed immunohistochemically and by Western blotting. For immunostaining, muscle frozen-sections preblocked with TBS-10% skim milk were incubated with one to four unique phages (5 x 10^{10} cfu each in 100 µl TBS-10% skim milk) simultaneously for 12 h at room temperature. After washing twice with TBS-T for 15 min, the sections were incubated with 1,000-fold-diluted anti-M13 mouse IgG (Progen Biotechnik, Heidelberg) in TBS-5% skim milk for 1 h. After washing twice as above, the sections were stained by using an Envision-HRP kit (Dako) according to the manufacturer’s protocol. For Western blotting, muscle sections (about 25-50 µg as protein) were dissolved in a 2-fold-concentrated sample buffer of SDS-PAGE (11), electrophoresed on 10% preparative gel, and transferred to a nitrocellulose membrane. The membrane was cut into strips 1-3 mm wide, blocked with 10% skim milk in TBS for 1 h, and incubated with scFv phages in TBS-10% skim milk for 2 h. In another set of experiments (with Tween treatment), the membrane strips were incubated with TBS-0.1% Tween 20 for 1 h, followed by blocking and phage incubation as described above, but in the presence of 0.1% Tween 20 (12). After washing with TBS-0.1% Tween 20 for 10 min six times, the strip was incubated with 10,000-fold-diluted anti-M13 mouse IgG-HRP conjugated (Amersham Pharmacia Biotech, USA) in TBS-0.1% Tween 20 for 1 h. After washing with TBS-0.1% Tween 20 as above, the blot was visualized with an ECL Plus™ Western blotting detection system (Amersham Pharmacia Biotech).

Two-dimensional (2-D) Western blotting and mass fingerprinting analysis. Muscle sections (about 30 and 300 µg as protein for Western and mass analysis, respectively) were dissolved in 250 µl of
a 2-D gel electrophoresis buffer (20 mM Tris base, 9 M urea, 2 M thiourea, 4% CHAPS, 65 mM dithioerythritol, and 0.5% IPG [immobilized pH gradient] buffer [Amersham Pharmacia Biotech]) and subjected to isoelectric focusing by using an IPGphor™ system (Amersham Pharmacia Biotech) with a 13 cm-long IPG strip (pH range, 3-10) according to the manufacturer’s protocol. The IPG strip was then applied to the top of an SDS-PAGE gel (7.5%, 14 x 12 cm). For mass fingerprinting analysis, proteins on the 2-D gel were visualized by using a copper-staining kit (BioRad, USA). The target protein spots were then cut out, in gel digested with trypsin, and analyzed with a MALDI-TOF (matrix-assisted laser desorption ionization-time of flight)-type mass-spectrometer (Voyager-DE™PRO, Applied Biosystems) (1, 13). The peptide mass fingerprinting analysis was performed by using the program MS-FIT. Western analysis was carried out as described above.

Immunoscreening of the human muscle cDNA expression library. A human skeletal muscle 5’-STRETCH PLUS™ cDNA library (Clontech, USA) was immunoscreened according to the manufacturer’s protocol by using scFv phages. Library plaques were transferred to nitrocellulose membranes, incubated with scFv phages (5 x 10¹⁰ cfu/ml, for 2 h), and visualized by using anti-M13 mouse IgG-HRP conjugated and ECL Plus™ Western blotting detection reagent (Amersham Pharmacia Biotech, USA) as described above.

Results

Optimal conditions for ISPS in muscle. ISPS is performed by 1) dissection and isolation of target microstructures from surrounding tissues on a tissue section, 2) incubation of the tissue section with an scFv phage library, 3) collection of targets, and 4)
selection of target-specific phages (Fig. 1). A new laser micro-
dissector was developed to cut out the microstructures under a micro-
scope (Fig. 2A). The dissector produced a rectangular space of variable size (1 to 56 µm) on a tissue section with a single shot of 
an ultraviolet laser beam at 266 nm (Fig. 2B). Tissues surrounding 
the target were widely burnt off by precisely controlling the laser 
power. Spaces that were 15 µm wide surrounding a 40 x 40 µm² square microfragment were usually produced in a muscle section by the dissector (Fig. 2C). As a source of scFvs, we employed a phagemid library, Griffin. 1 (see Experimental Procedures). The scFv sequences were constructed with synthetic human V-gene sequences of immunoglobulin, V_h and V_l (3, 8). When the phages were rescued by using helper phages derived from M13 (VCSM13), the V-gene was fused with the gene III of the M13 phage and expressed as an scFv at the tip of the phage particle (2). The Griffin. 1 library contained about 10^9 independent unique clones (9).

We first determined the optimal conditions for phage antibody reaction with muscle microfragments. Anti-human α-actin and anti-
human GFAP (glial fibrillary acidic protein) phages (designated Actin89 and GFAP53, respectively) were obtained from the Griffin. 1 library by the conventional panning method using polystyrene tubes (10). Actin89 phage was used for the evaluation of specific binding to muscle microfragments and GFAP53 phage for that of non-specific binding, since GFAP is an astrocyte-specific protein. After reacting these phages with a muscle section, 3-5 microfragments (40 x 40 µm² square) were collected and washed three times to eliminate contamination by phages from residual tissues other than the microfragments. The phages bound to the microfragments were eluted with 1.4% triethylamine and allowed to infect Escherichia coli TG1.
The phage titer was measured as the number of ampicillin-resistant colonies (colony forming unit, cfu). Skim milk was employed as a blocking agent because of findings in studies of muscle immunostaining with phage antibody in which 10% skim milk, but not 5%, worked well as a blocking agent. We checked the blocking effect of 10% skim milk on the non-specific binding of GFAP53 phage to muscle fragments (Table I). As seen, preincubation with 10% skim milk or its presence during phage incubation was optimal in preventing the binding of unrelated phage GFAP53 to muscle fragments. Elevated amounts of non-specific phage binding were only seen in the absence of the blocking agent throughout the experiment. Interestingly, the binding of Actin89 phage to muscle fragments was not affected either in the presence or absence of 10% skim milk (Table I).

We next checked the relation between input phage concentrations and amounts of phage binding to muscle fragments (Table II). As seen, the numbers of Actin89 phage bound to muscle microfragments were linearly related to the concentrations of input Actin89 phage ($10^8$-$10^{12}$ cfu/ml) under optimal conditions. Similar linearity was observed when Actin89 was reacted with purified $\alpha$-actin bound to an ELISA (enzyme-linked immunosorbent assay) plate (input $10^5$-$10^{12}$ cfu/ml, data not shown). When the input phage concentration was $10^{12}$ cfu/ml, the amount of Actin89 bound to the plate was 13,000 cfu/1000 $\mu$m$^2$. An anti-thyroglobulin phage was examined as an unrelated phage.

We also investigated the effect of the duration of incubation on the binding of Actin89 phage to a muscle microfragment. No significant difference was observed among the numbers of the phages collected after incubation for 3, 6, 12, and 60 hours. The antigen retrieval effect of solvents on muscle microfragments was evaluated
by quantifying the binding capacity of Actin89 to the microfragment. Among the solvents tested (acetone, methanol, ethanol, isopropanol, chloroform, sodium dodecyl sulfate [0.01 and 0.1%], Tween 20 [1%], and paraformaldehyde [2 and 4%]), acetone and ethanol pretreatment enhanced binding capacity the most, namely, three times more than in the absence of treatment. Acetone pretreatment was employed in the standard protocol for ISPS on human muscle.

**Screening of the Griffin. 1 library on a micro-area of muscle.**

We produced various size microfragments on a transverse section of human muscle by laser-dissection and incubated them with the Griffin. 1 library. The bound phages were directly eluted from microfragments and used to infect TG1 (‘direct colony recovery’ protocol), or the scFv sequences of bound phages were amplified by PCR and subcloned into phagemid vector pHEN2 (‘PCR-fragment subcloning’ protocol). Phages were rescued from ampicillin-resistant colonies, and after unique clones were picked up by Hae III-fingerprinting, their specificity for muscle antigens was checked by both immunohistochemistry and Western blotting of muscle antigens.

The PCR-fragment subcloning protocol yielded no phage clones from an area of 100 (10 x 10) µm² and 3 phage clones from 1,100 (33 x 33) µm². The 3 phage clones from the 1,100 µm² microfragment showed no specificity for muscle antigens, as judged by immunostaining. When the reaction area was enlarged to 4,800 µm² (3 microfragments of 40 x 40 µm²), we obtained a number of unique phage clones in two independent trials both by direct colony recovery and PCR-fragment subcloning protocols (Table III, experiments A and B). The PCR-fragment subcloning protocol gave us larger numbers of unique clones than the direct colony recovery protocol (Table III), since less
than 1 cfu of phagemid was amplified under our PCR conditions. About 8 phage particles were equivalent to 1 cfu (Furuta et al, unpublished result). The two trials, A and B, yielded three positive clones, A28, B6, and B85, which specifically reacted with 37, 100, and 42 kDa muscle antigens, respectively (Tables III, IV, Fig. 3A). In PCR fragment subcloning experiments, the collected phages (round 1) were amplified and applied to the second round of selection (round 2) as in the same manner. In experiment A, the positive clone obtained from round 1 (A28) was again recovered in round 2; however, in experiment B, the positive clone in round 1 (B85) was not found in round 2 (Table III). This suggested that repeated panning steps sometimes lost positive clones which were present in the earlier round of phage pool.

We planned another series of experiments by using a longitudinal muscle section to obtain a homogeneous distribution at the reaction surface of muscle antigens which localized in muscle-specific, repeating zone structures such as the A- and I-bands. The screening was also performed on 4,800 µm² microfragments by using the Griffin.1 library. Three independent trials (experiments C, D, and E) yielded scFv clones reactive with 210 kDa protein (C43 and E2) and 42 kDa protein (C22, D2, E6, and E30) by the PCR-fragment subcloning protocol (Tables III, IV). The results of one-dimensional (1-D) Western analysis using the representative phage clones (A28, B6, E2, and E6) are demonstrated in Fig. 3A. As seen, the reactivity of these scFvs was markedly enhanced by Tween treatment of the blot membranes (12). Since these scFv phages were selected on a frozen-section mildly fixed by acetone, they may have preferentially reacted with renatured antigens on the membrane. A considerable number of phages with low specificity that reacted with
multiple bands on muscle Western analysis were obtained through experiments A to E (data not shown).

**Identification of muscle antigens by using scFv phages obtained in ISPS.** Two-dimensional (2-D) Western blotting of muscle proteins was performed by using positive scFv phages, and their corresponding antigens were assigned on a Coomassie-stained 2-D gel (Fig. 3B, C, and D). The 42 kDa protein recognized by several clones (B85, C22, D2, E6, and E30) had a pI of 5.2 and was classified as \( \alpha \)-actin (Fig. 3B, ‘Ac’), which accounts for about 20% of the total protein in muscle (14). Similarly, the 210 kDa antigen had a pI of 5.8 and was classified as a myosin heavy chain (Fig. 3B, My), which weighed about 40% of the total proteins in muscle (14), although it was not efficiently displayed on our 2-D gel system when compared with the results of a previous researcher who used agarose gel for the first dimension (15). These assignments of the 42 kDa and 210 kDa spots were confirmed both by Western analysis of the same 2-D blots using anti-actin or anti-myosin heavy chain mouse monoclonal antibodies (data not shown). Clone A28 recognized a doublet protein spot of 36-37 kDa with a pI of 4.7, suggesting that it reacted with more than one isoform of the antigen (Fig. 3C). Clone B6 recognized a single spot of 100 kDa with a pI of 5.3 (Fig. 3D). These antigens were also assigned to spots on the 2-D gel (Fig. 3B, A28 and B6, respectively).

Since the scFv phages A28 and B6 were highly specific, immunoscreening of a human muscle expression library (cloned in \( \lambda \)TriplEx vector, Clontech Laboratories, USA) was performed to identify the corresponding antigens (Fig. 3E). We found 80 positive plaques for A28 out of \( 1.2 \times 10^4 \) \( \lambda \) clones and 3 positive plaques for B6 out of \( 1.2 \times 10^5 \) \( \lambda \) clones. For A28, four of the five \( \lambda \) inserts
had a sequence that matched the human cDNA sequence of tropomyosin-α (TPM3) 100%, and the other λ insert matched the other isoform of tropomyosin-α (TPM1). For B6, all three sequences of the λ inserts completely matched the human α-actinin 2 sequence. The observed molecular weights and pI values for the two protein spots were consistent with those calculated for tropomyosin-α (33 kDa and 4.6) and α-actinin 2 (104 kDa and 5.3).

To confirm the results of immunoscreening, the 37 and 100 kDa protein spots visualized by copper-staining (BioRad, USA) were cut out, digested in gel with trypsin (1), and subjected to mass fingerprinting analysis with a MALDI-TOF mass-spectrometer (13). The masses of the tryptic peptides obtained for the 36-37 kDa protein best matched those of α- and β-tropomyosin. Similarly, a mass fingerprint of the 100 kDa protein closely matched that of α-actinin 2.

Histochemical examination revealed that scFv phages A28 (anti-tropomyosin) and B6 (anti-α-actinin) diffusely stained muscle cells (Fig. 4). Anti-myosin heavy chain phage E2 stained muscle cells in an isoform-dependent manner (Fig. 4), and another anti-myosin heavy chain phage, C43, also showed an isoform-dependent staining pattern (data not shown). As expected, the isoforms of the myosin heavy chain recognized by the phage clones C43 and E2 were identified by immunoscreening to be the myosin heavy chains IIa and β (MYH7), respectively. By contrast, only one of the five clones of anti-actin scFv phage, E6, weakly stained the muscle cells, possibly because of the low affinity of anti-actin scFvs for F-actin. The features of the anti-muscle scFvs are listed in Table IV.
Discussion

We performed ISPS on a 4,800 \( \mu \text{m}^2 \) microarea of muscle. The 40 x 40 \( \mu \text{m}^2 \) area corresponded to about 0.5-1.0 transverse sections of muscle cells (see Fig. 2C). A single round of selection was fruitful in all of our trials (experiments A to E) to obtain specific scFvs against minute amounts of antigens present in a micro-area, resulting in the identification of the muscle proteins by immunoscreening. Most of the specific scFv phages obtained here were for major muscle antigens, i.e., myosin heavy chain, \( \alpha \)-actin, and tropomyosin-\( \alpha \), present in the reaction area. Myosin and actin are the two major constituents of muscle and account for about 40% and 20%, respectively, of the total weight of muscle protein (14). Tropomyosin(s) is the third major protein in muscle (accounting for about 5% of the total [14]). Since the total amount of proteins in a 4 mm\(^2\) muscle section that was 2 \( \mu \text{m} \) thick was measured 2.5 \( \mu \text{g} \) (n=4), the amount of tropomyosin in 4,800 \( \mu \text{m}^2 \) microfragment was 150 pg; therefore, 31 fg = 6 x 10\(^5\) molecules of tropomyosin were estimated to exist in 1 \( \mu \text{m}^2 \) microfragments (2 \( \mu \text{m} \) thick). To our surprise, we also succeeded in obtaining an scFv phage against \( \alpha \)-actinin 2, which accounts for only 1% of muscle protein (14); hence, the total weight in 4,800 \( \mu \text{m}^2 \) is 30 pg (similarly, 6.3 fg = 4 x 10\(^4\) molecules of \( \alpha \)-actinin/\( \mu \text{m}^2 \) microfragments). The reason why the scFv for such a minor component (\( \alpha \)-actinin 2) was selected may have been the strong antigenicity of the antigen or the high abundance of anti-\( \alpha \)-actinin scFv(s) in the Griffin. 1 library. Another possibility is that a portion of the Z-disk, in which \( \alpha \)-actinin is localized (16), was efficiently exposed to the reaction surface on the transverse sections.
To select scFvs against minor antigens existing in target structures, a single round, rather than multiple rounds, of selection was recommended, since positive clones could be lost during repeated rounds of selection. Presumably, changes in phage population in a phage pool through the steps of panning are affected not only by the reactivity of scFvs displayed on the phage particles but also by biological factors such as the efficiency of phage production or the growth rates of *Escherichia coli* in each clone. High throughput analysis of the initial round of clones, which involves picking up unique clones from a large pool of redundant colonies and producing each clone phage, is a key for the further advance of ISPS.

We noted the high quality of the phage antibodies (scFvs) selected against such minute amounts of antigens in situ from the Griffin.1 library. Among the phage antibodies obtained here, anti-myosin heavy chain phage antibodies are definitely isoform(s)-specific on immunohistochemistry (Fig. 4) and immunoscreening, indicating that they are reliable for immunological examinations. As expected, many of the antibodies obtained by ISPS in this study can be used for Western analyses, immunohistochemistry, and immunoscreening (Table IV). Interestingly, the obtained phage antibodies showed higher reactivity against renatured antigens (Fig. 3A), suggesting that 'conformation-dependent' phage antibodies can be selected by ISPS. Thus, phage antibodies that are useful for a wide variety of immunological and biochemical analyses of the target antigens could be obtained by ISPS in combination with the Griffin.1 library. Theoretically, ISPS can also be applied to structures consisting of non-protein components, such as complex sugars and lipids. When monoclonal antibodies are obtained against non-protein
components, the antigens can be identified by other methods besides immunoscreening, for example, by immunoaffinity purification.

A major technical problem encountered in this study was that the collected target fragments tended to stick to the inside of glass capillaries. This was partially prevented by the addition of detergent to the buffer overlaid on tissues or by careful manipulation of the capillaries. Siliconization of capillaries was not effective to solve this problem. In this case, fragments collected from paraffin sections were not very sticky to glass capillaries. When the target structures were very small (less than 10 µm), we found another problem, namely that substantial amounts of the collected targets were lost during washing by centrifugation. This problem remains to be solved. On the other hand, when the target structures were rather large (100 µm or more), no such problems were encountered through the experimental steps. Relatively large targets could be collected directly into PCR reactions by using a needle under a stereomicroscope after tissue sections were washed on glass and dried. In this method, it was not necessary to overlay a buffer on tissue sections during collection or to wash the collected targets by centrifugation.

There are four factors that may affect the sensitivity of ISPS: 1) the avidity of scFv phages for their target antigen (which depends on the Kd and the valency of the displayed scFvs), 2) the phage concentration, 3) the amount of antigens involved in the reaction, and 4) the reactivity of the antigens (affected by treatment of tissue section, such as by fixation, antigen retrieval treatment, drying, or others). The proteins in paraffin sections, the most common source of tissue samples, seemed to be less reactive with scFvs, possibly due to the lower reactivity of the antigens
(data not shown). It is important to find an effective method of antigen retrieval for a variety of histopathological structures when paraffin sections are used for ISPS. The scFv library can be modified to further improve the sensitivity of ISPS, since higher concentrations of individual phages are needed to detect lower amounts of antigens. Libraries with different diversities may be applied to a target structure depending on the amount of antigens to be identified.

In the last decade, applications of mass spectrometry (MS) to peptide profiling (17), protein profiling (18), and imaging of protein distribution (19) in tissue samples have been developed. The literature does not describe the amount of tissue needed for MS-based protein identification, although as little as 5 ng of protein has been identified by MS after separation on 2-D polyacrylamide gel electrophoresis (1). It should be pointed out, however, that ISPS was the only approach that enabled us to obtain highly specific monoclonal antibodies by using such a minute amount of tissue components. We were able to collect more than 100 target microstructures in one day, depending on their abundance in a tissue section. ISPS may enable the identification of even minor components of bodies, plaques, or micro-deposits of unknown components in various organs or subcutaneous lesions in focal and systemic disorders. Some of microstructures seen in neuromuscular and systemic disorders are under investigation.
Acknowledgements

We thank Dr. Greg Winter (Medical Research Council, Cambridge, UK) for providing us the Griffin. 1 library and useful comments on this work. We also acknowledge R. Kamata, K. Inugami, M. Shin, and D. Nishikiori for technical assistance. We are grateful to Drs. I. Nishino and I. Nonaka, NCNP, for arrangement of muscle samples, and Drs. S. Takashima, M. Imamura, and T. Sasaoka, NCNP, for helpful suggestions.
References


Footnotes

*This work is supported by grants from the Ministry of Health, Labor and Welfare and the Ministry of Education, Culture, Sports, Science and Technology (CREST and grant-in-aid for scientific research), Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1The abbreviations used are: ISPS, in situ phage screening; Fv, variable fragment of immunoglobulin; scFv, single chain Fv; V\text{H}, heavy chain variable region; V\text{L}, light chain variable region; cfu, colony forming units; GFAP, glial fibrillary acidic protein; PCR, polymerase chain reaction.

2Details of the Griffin. 1 library and phage protocols are also given on the Winter group home page (http://www.mrc-cpe.cam.ac.uk/phage/index.html).
Figure legends

Figure 1  Schematic presentation of in situ phage screening (ISPS). ISPS is performed by 1) laser-dissection and isolation of target microstructures from surrounding tissues on a tissue section, 2) incubation of the tissue section with an scFv phage library, 3) collection of targets, and 4) rescue of the bound phages and selection of target-specific phages. The scFv sequences of phages bound to microfragments were amplified by PCR (Fr) and subcloned into phagemid vector pHEN2 (See Experimental Procedures). No PCR products were obtained from wash supernatant of the microfragments (Sup), indicating that the bound phages were not contaminated by those from residual tissues other than the microfragments.

Figure 2  A, A diagram of a new laser micro-dissector constructed by combining a UV LASER SYSTEM HCL-2100SUV (HOYA Continuum CO., LTD., Japan) and an inverted research microscope IX70 (Olympus Optical CO., LTD., Japan). 1, UV laser head; 2, slit; 3, dichroic mirror; 4, halogen lamp; 5, objective (UV laser); 6, sample slide; 7, objective (observation); 8, prism (light path switch); 9, CCD camera. B, Square spaces burnt in a muscle frozen-section by the dissector. Scale bar represents 10 μm. From the top: 1, 3, 10, and 20 μm squares. C, A 40 x 40 μm square muscle microfragment fractioned in a frozen section with the dissector. The section was stained with hematoxylin to demonstrate the architecture of muscle. Scale bar represents 40 μm.

Figure 3  Identification of human muscle antigens by in situ phage screening (ISPS). A, The 1-D Western analysis of muscle proteins by the representative scFv phages. About 2 μg of human muscle protein on each strip was reacted with scFv phages with (lane 1-4) or without (lane 5-8) Tween treatment. P, proteins on the strip.
visualized by colloidal gold stain (BioRad). Lanes 1 and 5, phage A28 ($5 \times 10^8$ cfu/ml); lanes 2 and 6, phage B6 ($3 \times 10^{10}$ cfu/ml); lanes 3 and 7, phage E2 ($5 \times 10^9$ cfu/ml); lanes 4 and 8, phage E6 ($3 \times 10^{10}$ cfu/ml). B, Coomassie stain of human muscle proteins ($60 \mu$g) electrophoresed on a 2-D gel. Proteins identified by ISPS in this work are indicated by arrowheads: My, myosin heavy chain; Ac, $\alpha$-actin; A28, the antigen recognized by phage A28 (tropomyosin, Tm); B6, the antigen recognized by phage B6 ($\alpha$-actinin, An). The pH range in the first dimension (isoelectric focusing) is shown at the bottom. C and D, The 2-D Western analysis of human muscle proteins ($30 \mu$g) by using phages A28 (C) and B6 (D) at a concentration of $5 \times 10^{10}$ cfu/ml. E, Immunoscreening of a muscle expression library with phage A28. The $\lambda$-plaques of the secondary screening were reacted with phage A28 ($5 \times 10^{10}$ cfu/ml). Positive plaques are indicated by arrows.

**Figure 4** Immunostaining of human muscle sections by using scFv phages B9 (negative phage), A28 (anti-tropomyosin-$\alpha$), B6 (anti-$\alpha$-actinin 2), and E2 (anti-myosin heavy chain $\beta$, longitudinal section).
Table I

Effect of skim milk on specific or non-specific phage binding to muscle microfragments.

<table>
<thead>
<tr>
<th>Skim milk</th>
<th>Phage recovery (cfu/1000 μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Actin89</td>
</tr>
<tr>
<td>Preblock</td>
<td>Incubation</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Muscle microfragments were incubated with or without 10% skim milk for 2 h (preblock) and incubated with Actin89 (10^{10} cfu/ml) or GFAP53 (10^{12} cfu/ml) phages with or without 10% skim milk for 12 h (incubation). Values represent mean ± SD (n=3). ND, not determined.
Table II
Relation between input phage concentration and phage recovery from muscle microfragments.

<table>
<thead>
<tr>
<th>Phage input (CFU/ml)</th>
<th>Phage recovery (CFU/1000 μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Actin89</td>
</tr>
<tr>
<td>$10^{12}$</td>
<td>850 ± 300 *</td>
</tr>
<tr>
<td>$10^{11}$</td>
<td>73 ± 31 *</td>
</tr>
<tr>
<td>$10^{10}$</td>
<td>15 ± 14 *</td>
</tr>
<tr>
<td>$10^{9}$</td>
<td>0.8 ± 0.4 **</td>
</tr>
<tr>
<td>$10^{8}$</td>
<td>0 **</td>
</tr>
</tbody>
</table>

Values represent mean ± SD; n=5(*) or 3(**). ND, not determined.
Table III
Profile of *in situ* phage screening on 4,800 μm² of muscle microfragments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Number of clones</th>
<th>Analyzed</th>
<th>Unique</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PCR round 1</td>
<td>72</td>
<td>29</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PCR round 2</td>
<td>24</td>
<td>8</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct</td>
<td>22</td>
<td>14</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PCR round 1</td>
<td>72</td>
<td>47</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PCR round 2</td>
<td>72</td>
<td>23</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>PCR</td>
<td>48</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>D</td>
<td>PCR</td>
<td>48</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>E</td>
<td>PCR</td>
<td>48</td>
<td>9</td>
<td>3</td>
</tr>
</tbody>
</table>

Clones were selected from the Griffin. 1 library on transverse (A and B) or longitudinal muscle sections (C, D, and E) by a direct colony recovery protocol (Direct) or a PCR-fragment subcloning protocol (PCR).
Table IV
Features of anti-muscle scFv clones.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Western</th>
<th>Antigen</th>
<th>IHC</th>
<th>IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>A28</td>
<td>37 kDa</td>
<td>tropomyosin-(\alpha)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B6</td>
<td>100 kDa</td>
<td>(\alpha)-actinin 2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B85</td>
<td>42 kDa</td>
<td>(\alpha)-actin</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>C22</td>
<td>42 kDa</td>
<td>(\alpha)-actin</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>C43</td>
<td>210 kDa</td>
<td>myosin heavy chain IIa</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D2</td>
<td>42 kDa</td>
<td>(\alpha)-actin</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>E2</td>
<td>210 kDa</td>
<td>myosin heavy chain (\beta)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E6</td>
<td>42 kDa</td>
<td>(\alpha)-actin</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>E30</td>
<td>42 kDa</td>
<td>(\alpha)-actin</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

IHC, immunohistochemistry; IS, immunoscreening; ND, not determined.
Tanaka T. et al. Figure 1, TOP

1) Laser dissection
   Tissue section

2) Micropipette
   scFv phage library

3) Tissue microfragment

4) Fr Sup
   PCR
   Clone analysis
   Western blot
   Histochemistry
Tanaka T. et al. Figure 2
TOP

A

B

C
Tanaka T. et al. Figure 4, TOP

B9 (negative)  A28 (tropomyosin)  B6 (α-actinin)  E2 (myosin heavy chain)
In situ phage screening; a method for identification of subnanogram tissue components in situ
Torahiko Tanaka, Takashi Ito, Masaru Furuta, Chikashi Eguchi, Hiroyuki Toda, Eriko Wakabayashi-Takai and Kiyotoshi Kaneko

J. Biol. Chem. published online June 4, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203547200

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