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A novel excision selection method for isolation of antibodies binding antigens expressed specifically by rare cells in tissue sections

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ABSTRACT

There is a growing appreciation of single cell technologies to provide increased biological insight and allow development of improved therapeutics. The central dogma explains why single cell technologies is further advanced in studies targeting nucleic acids compared to proteins, as nucleic acid amplification makes experimental detection possible. Here we describe a novel method for single round phage display selection of antibody fragments from genetic libraries targeting antigens expressed by rare cells in tissue sections. We present and discuss the results of two selections of antibodies recognizing antigens expressed by perivascular cells surrounding capillaries located in a human brain section; with the aim of identifying biomarkers expressed by pericytes. The area targeted for selection was identified by a known biomarker and morphological appearance, however *in situ* hybridizations to nucleic acids can also be used for the identification of target cells. The antibody selections were performed directly on the tissue sections followed by excision of the target cells using a glass capillary attached to micromanipulation equipment. Antibodies bound to the target cells were characterized using ELISA, immunocytochemistry and immunohistochemistry. The described method will provide a valuable tool for the discovery of novel biomarkers on rare cells in all types of tissues.

INTRODUCTION

Cellular heterogeneity represents a major obstacle in the identification of biomarkers expressed by specific cell types. Furthermore, most conventional proteomics technologies do not allow analysis on the single cell level. Here we describe a novel technology allowing proteomic studies to be performed *in situ* targeting only a few cells. The technology relies on phage display selection of recombinant antibody libraries. The strength of phage display lies in the physical genotype-phenotype linkage provided by the phage particle, thereby allowing selection of genetic libraries based on the function of the encoded proteins. By in-frame cloning of a foreign DNA sequence with one of the phage capsid genes, the phage particle displays the foreign protein on its surface and carries the gene encoding the protein inside the particle (1). In general, phage display selections are performed in iterative three-step cycles; the phage displayed repertoire of antigen binding fragments (e.g. antibody fragments) are incubated with the antigen, followed by removal of non-binding phage antibodies through washing, finally the bound phage antibodies are eluted and amplified for the next round of selection. In the work presented here, we do not perform consecutive rounds of selection, but only one round of selection, before screening of the output monoclonal phage antibodies. In practice, the selections can be done in various ways depending on the type of target antigen. A review of *in vitro* selections with examples of antibodies with unique properties has been published by Geyer *et al*. (2). For more advanced selections, such as *in vivo* selections, a recent review focusing on the identification of antibody-targetable and accessible antigens in intact tumors has also been published (3).

Most often, when phage display has been applied as a discovery tool to identify specific biomarkers expressed by individual cell types, the target cells have been isolated and

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cultivated. However, it is generally accepted that removal of any cell from its native microenvironment will impact the gene expression, composition and modification of the antigens. Especially upon cultivation, it has been observed that the cells adopt a gene expression profile greatly impacted by the *in vitro* conditions (4–6). Hence, antibodies selected against cultured cells might not bind in tissue sections or *in vivo* applications. Methods for selection on a small number of cells or even a single cell have been developed (4–7). These methods can be applied for selection against single cells (e.g. from blood samples) or a few cells in their native environment (e.g. paraffin embedded or frozen tissue sections) with increased probability of finding antibodies recognizing authentic antigens present in the natural milieu of the target cells (8,9). However, the selection of antibodies against cells in tissue sections is still far from trivial, especially since target cells are normally scattered in the tissue. The methods developed so far allow selection on one cell or one target area per tissue section and are therefore not ideally suited for selection of multiple cells or target areas on the same tissue section. Other methods relying on the isolation of cells by use of laser capture microdissection (LCM) has proven less optimal in combination with phage library selections, due to problems of retrieving viable phage following selection (4,10,11).

When applying selection of phage antibody libraries as a discovery tool, it is essential that the number of selection rounds is kept to a minimum, preferably one, in order to ensure that there is a minimum of bias toward selecting antibodies recognizing highly expressed antigen or antibodies binding to a small subset of antigens with high affinity.

The antibodies resulting from such selections are nevertheless extremely valuable tools in the identification and characterization of rare cells like stem cells or circulating tumor cells; and together with nucleic acid based biomarkers they will allow for deeper genomic, transcriptomic and proteomic analysis even down to the level of single cells (12,13). Additionally, specific antibodies could be used for targeting RNAi therapeutics and other types of antibody drug conjugates to specific cells for higher efficiency (14–16).

In this paper we describe a method for single round selection of antibodies from phage-display libraries against clusters of rare target cells surrounding a capillary identified in all types of tissue sections. The technology is based on the precise excision of the area of interest after biopanning on the tissue section (Figure 1). The tissue excision is finely controlled by micromanipulation with a sharp glass capillary and the tissue of interest is collected by syringe-assisted micropumping. This allows for a very versatile selection on multiple small areas of different shapes in tissue sections. The method necessitates only a minimum of special laboratory equipment and training. The main requirement for a successful selection is the ability to identify small clusters of rare target cells in the tissue section. This can be done by immunohistochemistry, morphology or hybridizations. The method will be very useful for selection of antibodies for novel identification of cell populations or the further subdivision of heterogeneous cell populations. The method is particularly well suited for the selection of antibodies marking rare cells.

MATERIALS AND METHODS

Excision selection

The selections were performed on human frontal cortex sections (kindly provided by Professor Claudio Franceschi, University of Bologna, Italy) with the Predator library (17). Human cerebral cortex sections were snap-frozen and sampled in 8 microns sections on a cryostat. Prior to selections, cryostat sections were thawed at room temperature (RT) for 10 min and re-hydrated for 5 min in phosphate-buffered saline (PBS). The sections were subsequently fixed in 4% (w/v) paraformaldehyde (PFA) diluted in PBS for 12 min followed by 3 × 5 min wash in PBS. The tissue was blocked in PBS containing 2% (w/v) Marvel dried skimmed milk powder (MPBS) added 5% (v/v) goat serum (Sigma-Aldrich, Denmark) at RT for 1 h. After blocking, the tissue sections were incubated with the Predator library concurrently with Cy3 conjugated Anti-NG2 Chondroitin Sulfate Proteoglycan antibody (Merck Millipore, Denmark) diluted 1:100 in 2% (w/v) MPBS at 4°C overnight. For visualization of NG2 positive cells, the slides were incubated with 20 μg/ml Alexa 546 conjugated goat anti-Rabbit IgG (H+L) (Thermo Fisher Scientific) in 2% (w/v) MPBS at RT for 1 h. Slides were washed four times with PBS and mounted with Mounting Medium (Sigma-Aldrich). Pericytes were identified by NG2 staining in combination with assessment of morphology under an inverted microscope (Leica DMI3000 B, Leica Microsystems). The pericyte-covered capillaries were excised from the tissue with a micropipette (holding pipet, Origio) with a broken tip (for creating a sharp edge) controlled by micromanipulation equipment (Narishige, Model MM-188, Nikon). Navigating the sharp edge of the micropipette onto the border area of the capillary and gently scraping toward the center of the capillary loosened the target tissue from the surrounding tissue. Subsequently, the loosened capillary with bound phage antibodies was harvested by aspiration into the micropipette. Two capillaries were excised in each selection, and these were eluted into microcentrifuge tubes containing trypsin from Bovine pancreas (Sigma-Aldrich, Denmark) with a final concentration of 1 mg/ml and incubated at 37°C for 30 min followed by addition of 10% (v/v) fetal bovine serum. A total of 1 ml of pre-cultured *Escherichia coli* strain XL1-Blue at the logarithmic growth phase was transferred to the trypsinized phage solutions and incubated at 37°C for 30 min before plating on selective agar plates.

Phage production

Colonies were picked into Nunc 96-well Microtiter plates (Thermo Scientific) containing 200 μl 2xTY containing 100 μg/ml ampicillin and 1% (w/v) glucose and incubated in humidified boxes overnight at 37°C, 200 rpm. Phage antibody clones were following produced monoclonally in 96 well format by inoculating overnight culture into 200 μl fresh growth medium and grown for 2 h. Each well were superinfected with KM13 helper phages and incubated at 37°C, 200 rpm for 1 h. Bacteria were pelleted by centrifugation at 1800 *g* for 10 min. Pellets were subsequently resuspended in 200 μl 2xTY medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin be-

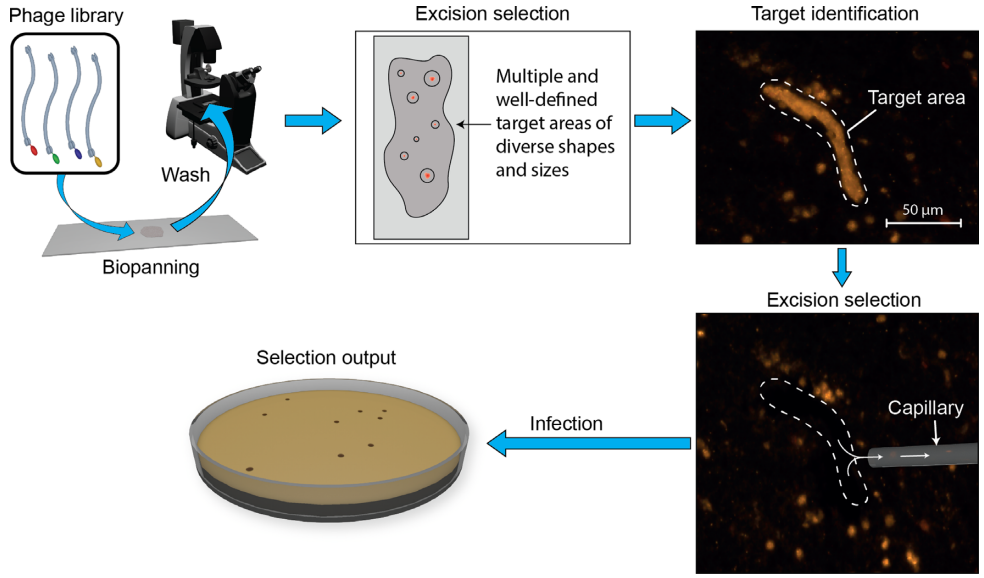

Figure 1. Outline of the excision selection method. Immunohistochemical or morphological identification of target cells was performed on the tissue section prior to biopanning. With excision selection the target area(s) can be excised from the section and harvested through aspiration followed by trypsin elution of bound phages. Trypsinized phages are rescued by infection into *Escherichia coli* and plated on a selective agar plate to allow for monoclonal colony formation. Images from one of the actual selections have been used to illustrate the before and after excision tissue section.

fore incubation overnight at 37°C, 200 rpm. The bacteria were pelleted by centrifugation at 1800 *g* for 30 min, and the supernatants transferred to new 96-well plates (Thermo Scientific). Phages were precipitated by adding 20% (w/v) polyethylene glycol 6000 (PEG 6000), 2.5 M NaCl and incubate overnight at 4°C. The precipitate was pelleted by centrifugation at 1800 *g*, 4°C for 30 min. Pellets were resuspended in appropriate volume of PBS. For large-scale phage production similar procedure was used in 50 ml cultures.

Eukaryotic cell cultivation

Eukaryotic cells were cultivated at standard conditions in T75 Tissue Culture Flasks (Sarstedt). Human Brain Vascular Pericytes (HBVP—Sciencell Research Laboratories), and Porcine Brain Vascular Pericytes (PBVP—Louiza Bohn Thomsen, Aalborg University) (18) were cultivated in Pericyte Growth Medium (Sciencell). Human Microvascular Endothelial Cells (HMEC-1—ATCC) and Human Brain Microvascular Endothelial Cells (ACBRI 376) (HBMEC—Cell Systems) were cultivated in Endothelial Growth Medium (Lonza). ASF2 fibroblasts (19), and hTERT adipose derived Mesenchymal stem cells (20) were cultivated in Dulbecco's Modified Eagle Medium (Lonza) with 1% penicillin-streptomycin and 10% (v/v) fetal bovine serum.

Expression and purification of antibody fragments

Antibody clones were expressed in *E. coli* strain LOBSTR (21) overnight in a shaking incubator (200 rpm) at 30°C. The cultures were centrifuged for 30 min at 3000 *g*, 4°C. The supernatants were filtered through 0.45 µm filters before protein precipitation by addition of ammonium sulfate to a final concentration of 30% (w/v) and incubated overnight at 4°C. Protein precipitate was pelleted by centrifugation at 15 000 *g*, for 15 min at 4°C. The pellets were resuspended in binding buffer (20 mM sodium phosphate, pH 7.0) and purified on protein A HP SpinTraps according to manufacturer's protocol (GE Healthcare Life Sciences).

ELISA

For whole-cell ELISA on the different cell lines (HBVP, PBVP, ASF2, hMscTert, HBMEC and HMEC-1) 10 000 cells of low passage number were seeded into each well of 96-well Cell Culture Cluster plates (Costar) and cultivated for 22–25 h for regeneration of surface antigens. Cells were washed twice in DPBS followed by fixation in 4% (w/v) PFA for 12 min at RT. After fixation, cells were rinsed with PBS and the wells blocked in 2% (w/v) MPBS for 2 h at RT. In the initial screenings phage antibodies were used by adding the prepared phages to the wells in 2% (w/v) MPBS and incubate for 90 min at RT. The wells were washed four times with PBS and subsequently incubated with 100 µl secondary HRP/Anti-M13 Monoclonal Conjugate antibody (GE Healthcare) diluted 1:3000 in 2% (w/v) MPBS for 1 h at RT. The wells were washed four times with PBS before adding the reaction substrate. For whole-cell ELISA with soluble antibody fragments the wells were incubated for 1.5 h at RT with 100 µl 2% (w/v) MPBS containing 10, 20 or 40 µg/ml of the antibody clones, respectively. The wells were washed two times with PBS prior to incubation with Anti-myc-HRP Antibody (Invitrogen) diluted 1:1000 in 2% (w/v) MPBS. The wells were washed four times with PBS before adding the reaction substrate. For the ELISA screenings against Fibronectin, 96-well Nunc MaxiSorp plates (Thermo Fisher) were coated overnight with 100 µl 2 µg/ml Fibronectin from human plasma (0.1% solution—Sigma-Aldrich) at 4°C. Wells were blocked with 2% (w/v) MPBS at RT for 2 h, and washed three times with 0.1% (v/v) Tween 20 in PBS (PBS-T). Each phage

clone was incubated in 2% (w/v) MPBS in dilutions series of 10^9, 10^{10}, 10^{11} and 10^{12} phages/ml for 90 min at RT. Unbound phages were removed in three washings steps with 0.1% (v/v) PBS-T before incubation with HRP/Anti-M13 Monoclonal Conjugate (GE Healthcare) diluted 1:5000 in 2% (w/v) MPBS. The wells were washed four times in 0.1% (v/v) PBS-T. All the ELISAs were developed using 3,3′,5,5′-Tetramethylbensidine (TMB) substrate solution (Sigma-Aldrich) and the reactions terminated by addition of 1M H_2SO_4. Absorbances were read at 450 nm and corrected with the reference wavelength 655 nm using a microplate reader (Bio-rad 550). ELISA was performed using 1 μM as highest concentration of rhPDGF Rβ (R&D systems) or rhNG2 (R&D systems) coated as a 3-fold dilution series in 96-well Nunc MaxiSorp plates. The coated protein was detected using 10 μg/ml of domain antibody followed by incubation with anti-myc-HRP antibody 1:1000 (Invitrogen). Two percent MPBS was used as control for background signal level. Protein coating was validated by detecting protein with anti-polyhistidine—HRP conjugated antibody (Sigma-Aldrich).

Immunocytochemistry

For immunocytochemistry (ICC), 50 000 target cells (HBVP, PBVP, hMscTert, ASF2, HBMEC and HMEC-1) were seeded in each well of an 8-well Nunc Lab-Tek Chamber Slide System and incubated overnight for attachment. The cells were rinsed with dPBS, fixed in 2% PFA for 15 min at RT and subsequently blocked in 2% (w/v) MPBS for 1.5 h at RT. For staining on HBVP only, the primary antibodies were diluted to a final concentration of 40 μg/ml in 2% MPBS and incubated overnight at 4°C. The next day, the wells were washed 2 × 5 min in PBS and incubated with a secondary anti c-Myc antibody (clone 9E10, Sigma-Aldrich) diluted 1:500 in 2% (w/v) MPBS, for 2 h at RT. The cells were washed 3 × 5 min in PBS and the secondary antibody was detected by an Alexa 488 conjugated Goat anti Mouse IgG antibody (Thermo Fisher Scientific). For assessment of antibody specificity though ICC on different cell types, the primary antibodies were diluted to a final concentration of 60 μg/ml in 2% MPBS and incubated overnight at 4°C. The wells were washed 2 × 5 min in PBS and the primary antibodies were detected by a monoclonal mouse Anti-c-Myc-Cy3 antibody (clone 9E10, Sigma-Aldrich) diluted 1:100. For both experiments, cell nuclei were stained using VECTASHIELD Mounting Medium with DAPI (Vector Labs, USA). Fluorescent images were obtained with a Leica DMI3000 B inverted microscope (Leica Microsystems, Germany).

Immunohistochemistry

Human frontal cortex sections (kindly provided by Professor Claudio Franceschi, University of Bologna, Italy) (22) were embedded in OCT cryo-protectant and sampled in 8 μm sections on a cryostat. Cryosections were thawed at RT, rehydrated and incubated in 0.3% Sudan Black B to quench the lipofuscin autofluorescence. The sections were subsequently washed three times in PBS to remove excess Sudan Black stain and blocked as described in the 'excision selection' section above. The tissue sections were incubated with combinations of the primary and secondary detection agents. NG2 was detected with Cy3 conjugated Rabbit anti-NG2 (Merck Millipore) diluted 1:100, endothelia was stained by biotinylated Ulex *Europeaus* Agglutenin I (Vector labs, USA) diluted 1:100 and detected with Cy3 conjugated streptavidin diluted 1:500 (Sigma-Aldrich). The selected antibody fragments were used at a concentration of 30 μg/ml. Mouse Anti-Myc Alexa488 conjugated antibody (AbD serotec) was used for detection of domain antibody. All primary incubations were made overnight at 4°C, while secondary incubations were made 2 h at RT. The sections were washed one time in PBS between primary and secondary and 3 × 5 min in PBS after incubation with secondary. Cell nuclei were stained using VECTASHIELD Mounting Medium with DAPI (Vector Labs, USA). Fluorescent images were obtained with a Leica DMI3000 B inverted microscope (Leica Microsystems, Germany).

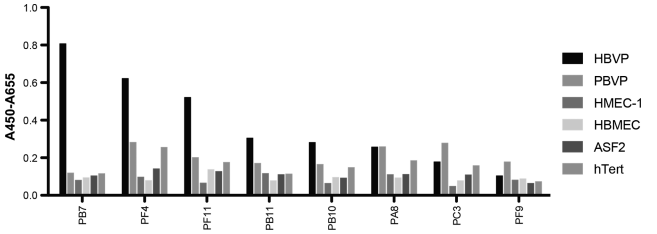

Figure 2. Whole-cell ELISA on relevant cell lines. The eight clones with highest preferential binding to pericyte cell lines were chosen for further evaluation. Five of these clones had highest signal on HBVP, and three had highest signal on PBVP.

RESULTS

The excision selection method was applied for the selection of antibodies binding antigens expressed by perivascular cells in human brain tissue sections. Two capillary-containing areas were excised, each one of these being considered as one independent selection. The two selections outputs comprised a total of 1150 antibody clones.

Following the single round of selection, the phage antibodies were grown and tested as monoclonal phage antibodies. An initial phage-antibody ELISA screening of 192 antibody clones on cultivated HBPV cells was made, and the phage antibodies resulting in low signals on cultivated HBVP cells were discarded (data not shown). Based on the initial screening, 53 antibody clones were selected for further analysis. In order to find antibodies with specificity toward the pericyte, ELISAs on HBVP, PBVP, ASF2, hMscTert, HBMEC and HMEC-1 cells were conducted. Of the 53 antibody clones screened, 5 had a binding preference for endothelial cells while 36 showed preference for pericytes (Table 1). Eight phage antibodies, which exhibited strongest and most specific binding to HBVP and PBVP, were chosen for further analysis (Figure 2). The antibodies, PB7, PF4, PF11 and PF10 showed highest signal on HBVPs, while PA8, PC3 and PF9 resulted in highest signal on PBVPs (Figure 2).

Fibronectin is known to be highly expressed by pericytes in capillaries and previous selections on cultivated HBVPs

Table 1. Results of screening for cellular specififcty

Cell type	Pericytes		Endothelial cells		Fibroblast	Mesenchymal stem cell
Cell line	HBVP	PBVP	HMEC-1	HBMEC	ASF2	hMscTert
No. of clones	25	11	3	2	2	10

Of 1150 phage particles eluted from the two selection, 192 were tested for binding to HBVPs. Of these 53 phage antibodies were tested for binding to various cell types (shown in table). Of these 53 clones 36 gave highest signal on pericytes, which indicates that ∼19% of the screened clones were preferentially marking the desired cell type after only one round of selection.

cells resulted in antibody clones binding fibronectin (23). To test for fibronectin binders, the eight clones were probed for affinity toward this antigen. A previously selected fibronectin dAb (C3) was included as a positive isotype control (23). The eight phage antibodies were tested against fibronectin in dilution series; five phage antibodies (PA8, PB7, PB10, PB11 and PF4) showed potential affinity for fibronectin (data not shown), as the signal increased in a concentration dependent manner analogously to the positive control C3.

While the fibronectin binders indicates a successful selection and potentially could prove extremely interesting, as antibodies against fibronectin has been shown to have therapeutic potential, we did not continue with these clones. The antibodies not binding fibronectin were chosen for further investigation to obtain novel biomarkers. The three clones (PC3, PF9 and PF11) were expressed and purified as soluble dAbs and applied in whole cell ELISA. Preferential binding of PF9 to the pericyte cells compared to HMEC-1 and HBMEC cells was confirmed, while the signals with PC3 and PF11 were too weak to give a clear indication of specificity (Figure 3).

Applying the antibodies in ICC on HBVP gave staining intensities that correlated with the signals obtained in the cell ELISA. Prominent binding was only observed for PF9 (C), whereas PC3 (A) and PF11 (B) did not vary significantly from the antibody control (Figure 4). Interestingly, the PF9 staining seemed to be mainly located at the cell membrane. To further exclude other known markers of pericytes an ELISA was made against NG2 and PDGF receptor β. There was no indication of binding of any of these markers with the PF9 antibody (data not shown).

For further characterization, the PF9 antibody was sequenced. The PF9 antibody is derived from the Predator library which only contain randomization in the CDR2 and CDR3 regions of the Vh domain. The sequence of CDR2 (DSYS) and CDR3 (VRSAWWT) show that the paratope is varied without any striking sequence pattern. Furhter, the PF9 antibody was applied in IHC on human frontal cortex sections. Co-staining was performed with a commercial anti-NG2 antibody in order to localize pericytes and agglutinin from Ulex *Europeaus* I, which marks endothelial cells (Figure 5). The staining pattern of PF9 (green, Figure 5A and C) partially overlaps that of the commercial anti-NG2 antibody (red, Figure 5B and C). For comparison the PF9 isotype control domain antibody does not give rise to specific staining (Figure 5D and F). Co-staining with Ulex *Europeaus* I agglutinin further indicates a perivascular localization of the PF9 antigen (Figure 5I).

ICC with PF9 was expanded to a panel of six different relevant cell types; HBVP, PBVP, HBMEC, HMEC-1, hMscTert and ASF2 cells (Figure 6). The ICC results revealed that PF9 binding to PBVPs was more prominent compared to the other cell types and it was further observed that the weak staining seen for HBMEC, ASF2 and hMscTert cells only appeared on a subset of the cells.

DISCUSSION

Only a few strategies for selecting genetic libraries of antibodies against small clusters of cells in tissue sections have previously been reported. Among the most promising strategies is the combination of LCM and phage display. A major drawback of this method is, however, the slide preparation conditions required for successful dissection, as these have been reported to compromise the phage infectivity and complicate the elution process of the phage antibodies bound to the dissected area (4,5).

The shadow stick selection method is another strategy that has successfully been applied to select binders to small clusters of cells (6,8). Here the nucleic acids constituting the genomes of the filamentous bacteriophages are exposed to UV light and crosslinks between nucleic acids prevents that the viral genome can replicate in bacteria. Phage particles protected from UV light contain a genome which can be replicated and translated, consequently conferring antibiotic resistance to the infected bacteria. However, the shadow stick method is limited by the boundaries of the shadow stick and selections on multiple and irregularly shaped areas in the tissue are not possible. This is problematic as antibody clones binding interesting antigens may not be located under the shadow stick and will therefore be lost. For irregular shaped clusters, the excision approach will ensure that more of the interesting tissue area can be targeted in the selection. Furthermore, the excision approach allows for the selection of multiple clusters within the same tissue section, whereas only one cluster can be shielded from UV irradiation in the shadow stick approach.

In the excision selection method, the selections can be performed on either cryo sections or deparafinized formalin fixated paraffin embedded sections. By using the excision approach, defined areas of the section is targeted with the purpose of identifying novel biomarkers specific for the targeted microenvironment (e.g. extracellular matrix proteins) or individual cells. As the selected area comprise a complex mixture of different antigens it is important to apply only a single round of selection. Performing multiple rounds of selections will generally favor antibodies that target highly expressed antigens or yield antibodies with high affinities (24,25). Unique biomarkers might not be present in high abundance nor may they be recognized by an antibody with high affinity. Thus, the most specific library variants will

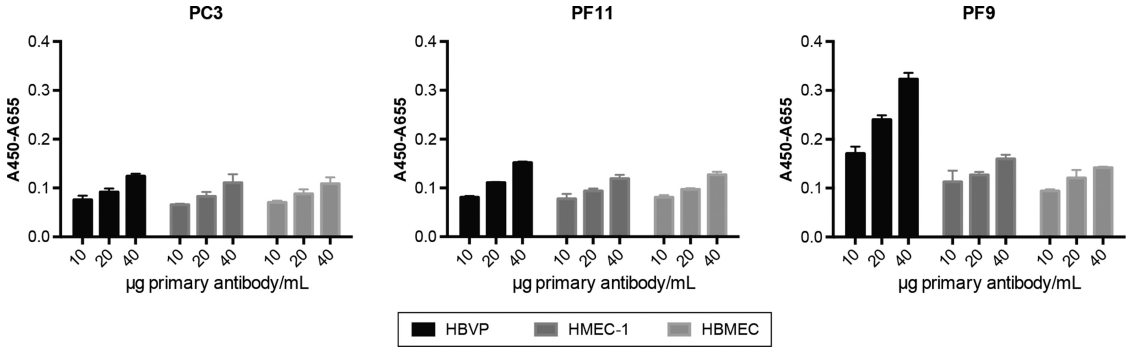

Figure 3. Whole-cell ELISA with purified soluble antibodies. PC3 and PF11 showed very low preferential difference in binding of HBVPs compared to the control cells. PF9 showed to be the most interesting when tested as soluble antibody, as binding to HBVP was more prominent compared to HMEC-1 and HBMEC cells

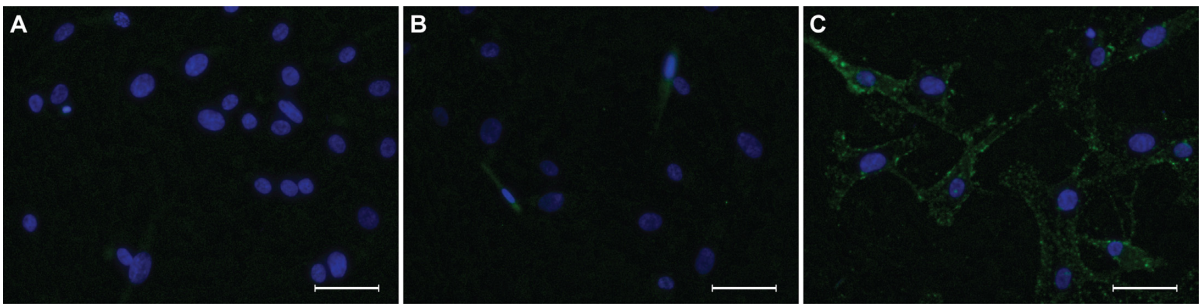

Figure 4. Immunocytochemistry (ICC) on HBVP. ICC staining intensities (in green) on HBVP with PC3 (**A**), PF11 (**B**) and PF9 (**C**) were in agreement with the whole- cell ELISA, since only PF9 gave rise to visible fluorescence signal, which seem to locate at the cell membrane. Cell nuclei were stained in blue by DAPI. Scale bar = 50 µm.

most likely be diluted or lost during subsequent amplification and selection steps.

Performing only one round of selection will normally result in an output of both phages displaying an antibody and non-displaying phages. This background can be reduced extensively by using a KM13 helper phage modified to be trypsin sensitive (26). Since the method described in this paper relies on a single round of selection, it is highly recommended to use this trypsin sensitive helper phage to reduce non-displaying background phages. However, when multiple rounds of selection are performed, the helper phage also increases the selection efficiency by ensuring that only relevant variants are amplified between selection rounds. Additionally, in selections performed with libraries constructed to include a trypsin cleavage site between the fusion protein and pIII, trypsin facilitates gentle and efficient elution of bound phage antibodies independent of their binding strength (17,27).

In order to increase the probability of selecting antibodies specific for the target cells, it is important that the area occupied by target cells only represent a small proportion of the tissue section. Then the non-target cells in the tissue section will absorb the vast majority of phage antibodies recognizing commonly expressed epitopes. It is well known that only 1–10% of the phage particles in a library preparation will be displaying an antibody (17,28). For optimal selections it is however important that the diversity of the genetic library is covered by a at least 100-fold excess of antibody displaying phages, this means that in order to cover a genetic library with a diversity of 10^8 unique antibody clones, 10^{11}–10^{12} phages should be applied for the selection to have 100 copies of each clone.

Before selection, the target cells needs to be identified. In some cases this can be done by morphological assessment or by using a known marker (e.g. an antibody against a known protein or *in situ* hybridization to genes, mRNA or microRNA). When the target cells have been identified the excision selection can be made on the tissue section or alternatively on the corresponding cells of a consecutive neighboring tissue section. A prerequisite for this approach is that identification of the target area on the selection slide can be made between sections. Typically, this requires hematoxylin or DAPI staining of the sections. The target cells needs to be rare in the section of interest, preferably with a ratio of 1–10 000 or more to background cells. The target cells do however not need to be a rare cell type in the organism as long as it is rare in the tissue section used for the selection.

The excision selection method holds a number of advantages compared to conventional strategies for selection of genetic libraries; (i) the use of tissue sections in selections increases the probability of discovering biomarkers with more relevance for histological analysis in the clinic, (ii) tissue sections also presents the opportunity to identify unique or up-regulated biomarkers expressed in the cells residing in specific microenvironments, (iii) the targeting of only a few cells within a tissue section predominantly occupied by irrelevant background cells reduce the number of output clones while improving the ratio of specific clones. On the contrary, se-

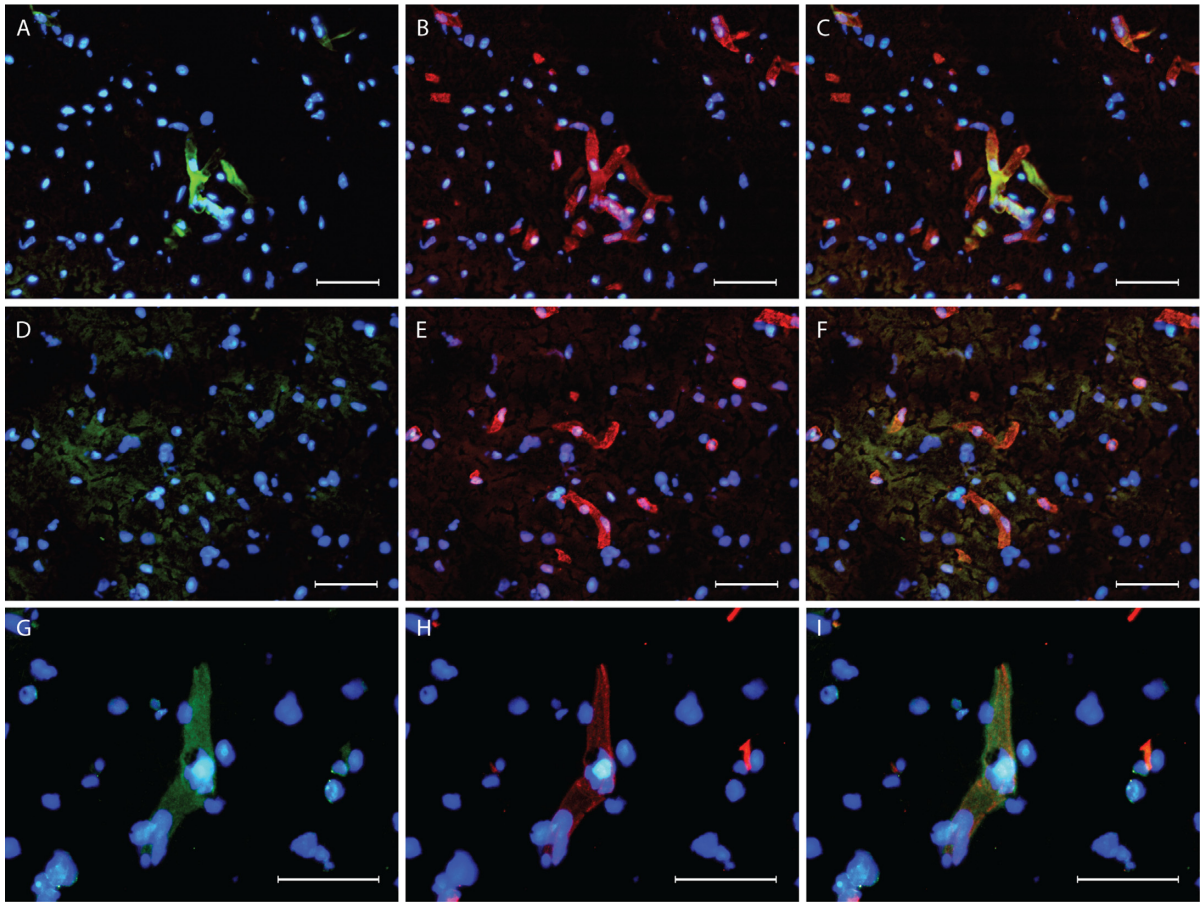

Figure 5. Immunohistochemical staining on human brain tissue sections. PF9 staining in green (**A** and **C**) and staining with CC17 isotype-matched antibody in green (**D** and **F**) were used in co-stainings with commercial anti-NG2 antibody in red (**B**, C and **E**, F, respectively). Staining with PF9 in green (**G** and **I**) resembled that of the Ulex *Europeaus* agglutinin staining in red (**H** and I) confirming the perivascular location of the PF9 antigen in the tissue. Cell nuclei were stained in blue by DAPI. Staining was performed on multiple sections and the pictures shown are representative for the staining observed on independent slides and throughout the sections. Scale bar = 50 μm.

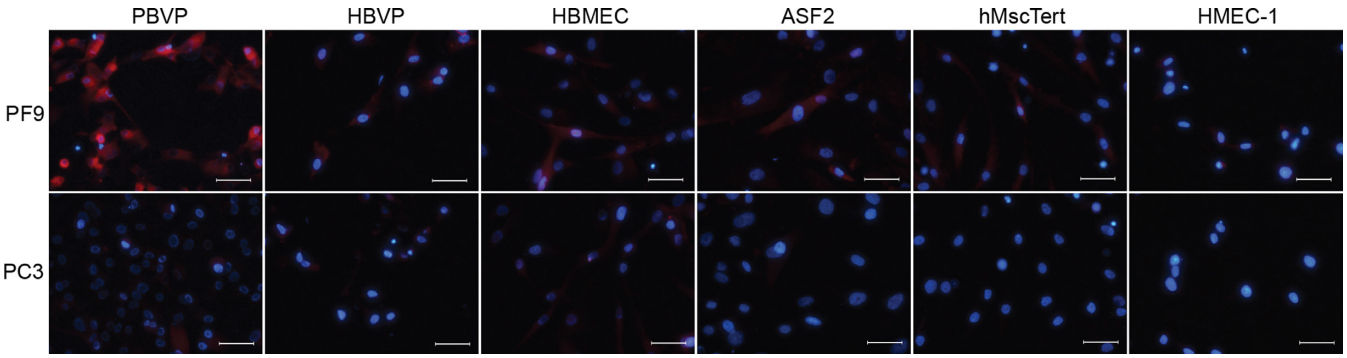

Figure 6. ICC for visualization of antibody specificity. ICC stainings were made on various relevant cell types to validate the specificity of PF9 (in red). The isotype-matched antibody PC3 (in red) was included for comparison. PF9 showed the most prominent binding to PBVP, and only a vague fluorescence was observed for the other cell types. Cell nuclei were stained in blue by DAPI. Scale bar = 50 μm.

lections performed on whole cell populations tend to be biased toward highly expressed and common antigens across the cell population. Whether the excision selection will favor high or low abundant antigens is mostly dependent on the exclusiveness of the antigen in the area of interest.

We applied the excision selection strategy to obtain antibody fragments showing specific binding to human vascular pericytes. The pericyte was considered a suitable target candidate due to the restrictive localization around microvessels and the clear need for pericyte-specific biomarkers. Such biomarkers are essential for further characterization of this highly heterogeneous cell type's functions, and implications in health and disease (29). Furthermore, antibodies selected against pericytes might prove valuable for

vascular-targeting by antibody drug conjugates or delivery of RNAi. The strength of the deployed selection method is the ability to target cells in their natural microenvironment. This retains pericyte heterogeneity and naturally occurring antigens. Antibody clones were selected based on their binding to antigens on cells associated with capillaries in human brain tissue. Two capillaries were excised from the surrounding tissue, and phage antibody clones bound to the excised areas were recovered by infection of *E. coli*. The selection output of 1150 clones is higher than the number of output clones obtained by the shadow stick selection technology on single cells in suspension (9) but comparable when considering the larger area of tissue section retrieved (8). For both shadow stick and excision selection on tissue, the expected yield is around 1–5 cfu per cell depending on the tissue, this number is also in good agreement with what has been observed when phage antibody libraries has been used to select for antibodies capable of mediating internalization (30). This means that if a cell cluster of 100 cells were excised in a selection, this would result in an expected output of 100–500 cfu.

From the two conducted selections 1150 antibody clones were recovered. Initially 192 clones were screened, from where 53 clones showed affinity for cultured human pericyte cells and 36 of these clones gave rise to higher signal on HBVP, or PBVP cells, indicating that pericyte antigens had been targeted in the selection (Table 1). However clones preferentially binding other cell types that would have been excised along with the pericytes e.g. five clones preferentially binding endothelial cells had also been selected. In subsequent screenings, several of the clones preferentially binding pericytes showed affinity for fibronectin, which is a marker that has previously been associated with pericyte biology (31,32). The selection of fibronectin binders corroborates the validity of the method. Part of this high occurrence of fibronectin binders could however also reflect the high secretion of this protein from the HBVP cell line used in the initial screening for binding to pericytes (23). Although only a part of the selection outputs were screened, these were considered representative for the total outputs.

The ELISA screenings on cells where done without permeabilizing the cells. As the selections were done in tissue sections, intracellular proteins would be accessible and a large part of the selection outputs would therefore be expected to show affinity for intracellular proteins. In this study, it was chosen to maintain cell membrane integrity in the ELISA screenings as therapeutic antibodies and antibodies for targeting and delivery should preferably target extracellular antigens (33,34).

The clones not binding fibronectin were tested in ELISA as soluble antibody to exclude artefacts from phage fusion protein (Figure 3). This ELISA confirmed binding specificity of the clone PF9 to the pericyte cells. That PC3 and PF11 failed to give a clear signal above the other cell lines could indicate that these clones binds less abundant antigens, which are not detectable without the amplification of signal stemming from the phage particle and the possible avidity from the display ratio on the phage. ICC staining with the soluble antibodies is in line with the ELISA results and further shows that the PF9 antigen is located at the cell membrane, indicating a membrane bound antigen (Figure 4). At this point PF9 was additionally tested for binding to PDGF receptor β and NG2, as these two proteins are some of the commonly used membrane bound markers for pericyte identification (35). No binding of these two antigens were observed with the PF9 antibody (data not shown)

The PF9 clone was applied for staining on the same human brain tissue material as used in the selection (Figure 5). The stainings show that PF9 is able to bind pericytes in cryo sections, and further indicate that the expression of the antigen for PF9 is located to the same regions as NG2 but are more restricted than that of NG2 (Figure 5C). The staining pattern of PF9 in combination with Ulex *Europeaus* agglutinin was made to further validate the perivascular localization PF9 in the tissue (Figure 5I). The PF9 and PC3 clones were tested as soluble antibodies against a panel of cell lines (Figure 6). Here the PF9 clone binds PBVPs with a higher intensity than HBVPs despite selection in human tissue. This may however be explained by differences in expression levels of the cognate antigen between the two cell lines. The HBVP cells have been isolated based on their expression of α-smooth muscle actin, and are therefore more homogeneous. The PBVP cells were freshly isolated by mechanical selection (18). It is therefore likely that the PBVPs are more heterogeneous and perhaps less differentiated. Alternatively, the antigen could also be shielded by the much denser layer of fibronectin secreted by the HBVP compared to the PBVP cells.

When using very rare material for the antibody-phage selection of antibody clones, the screening obviously poses a challenge, as additional material is needed for this. As described in this paper the initial screenings can be performed on cultured cell lines to test for specificity before qualitatively assessing the clones on the scarce selection material e.g. human tissue sections.

Alternatively the presence of around 1000 clones after the excision selection in tissue is easily managed by high-throughput sequencing. Next generation sequencing methods allow sequencing of up to millions of inserts in parallel, and have gained popularity as a screening method of large antibody repertoires. This way the entire selection output can be sequenced and clones appearing more than expected in the selection output can be studied in detail for binding specificity (36,37).

Also various high content screening platforms could easily be used for the initial qualitatively screening of clones in the numbers expected with the described selection method (38). Finally the selection output could also be reduced further by setting up more stringent wash/selection conditions or reducing the number of cells excised; this will however be subject evaluation based on the individual selection material at hand. The identification of the target molecule is of course central for the further development of selected antibodies. Target identification is however by no means a trivial task. In the case of PF9 the ELISA signals are relatively low indicating that the antigen is expressed at a low level or that the affinity of the antibody is not high. This makes antigen identification difficult for the PF9 clone. That PF9 would bind a low abundant antigen would however not be surprising given the selection setup, and the fact that other clones binding the highly abundant antigen fibronectin have already been discarded.

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