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Identification of Novel Macropinocytosing Human Antibodies by Phage Display and High-Content Analysis

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Abstract

Internalizing antibodies have great potential for the development of targeted therapeutics. Antibodies that internalize via the macropinocytosis pathway are particularly promising since macropinocytosis is capable of mediating rapid, bulk uptake and is selectively upregulated in many cancers. We hereby describe a method for identifying antibodies that internalize via macropinocytosis by screening phage-displayed single chain antibody selection outputs with an automated fluorescent microscopy-based High Content Analysis platform. Furthermore, this method can be similarly applied to other endocytic pathways if other fluorescent, pathway-specific, soluble markers are available.

Keywords

Macropinocytosis; phage antibody display; endocytosis pathway; high-throughput screening; high content analysis; human monoclonal antibodies; cancer metabolism; cancer cell membrane dynamics

1. Introduction

There is currently much interest in utilizing internalizing antibodies as the basis for developing targeted therapeutics including antibody-drug conjugates (Behrens, C. R. and Liu, B., 2014; Carter, P. J. and Senter, P. D., 2008; Scott, A. M., et al., 2012; Sherbenou, D. W., et al., 2015). The uptake efficiency and intracellular fate of delivered agents will affect therapeutic potency and depends on the internalization pathway utilized. Macropinocytosis is a form of bulk uptake that permits rapid and efficient internalization of antibody-delivered cargos (Ha, K. D., et al., 2014). Additionally, macropinocytosis is selectively up-regulated in many cancer types. For example, Ras-transformed pancreatic cancer cells upregulate macropinocytosis to increase amino acid uptake (Commisso, C., et al., 2013). Thus, antibodies capable of efficient internalization via macropinocytosis are intriguing candidates for development into targeted therapeutics, particularly against cancer.

Given the existence of multiple pathways of internalization, robust and efficient methods for identifying macropinocytosing antibodies will greatly aid in their discovery and translation

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into therapeutics. To address this need, we developed a high-throughput, high content analysis (HT-HCA) screening protocol that employs automated fluorescent microscopy-based analysis to identify phage antibodies that colocalize with Texas Red-conjugated 70 kDa neutral dextran (ND70-TR), a macropinocytosis marker (Ha, K. D., et al., 2014). We hereby provide a detailed description of this protocol, including phage antibody identification, validation of macropinocytosis with full-length human IgG, functional internalization based on payload delivery, and identification of receptors bound by novel macropinocytosing antibodies.

2. High-Throughput, High Content Analysis of Macropinocytic Antibodies: The Method

This HT-HCA of novel antibodies from phage libraries largely depends on fluorescent colocalization. Using either standard or confocal fluorescent microscopy techniques, two standard correlation coefficients, called the Pearson colocalization coefficient (PCC) or the Manders overlap coefficient (MOC) can be used to quantify the degree of colocalization between two distinct, fluorescent sources both *in vitro* and *in vivo* (Adler, J. and Parmryd, I., 2010; Huang, S., et al., 2015). PCC largely relies on degrees of spatial overlap while MOC additionally considers fluorescent intensities (Dunn, K. W., et al., 2011). One critical limitation to utilizing PCC and MOC for determining colocalization through fluorescent microscopy is that the typical optical resolutions of confocal microscopes tend to range between 200-300 nm, potentially leading to false positive colocalization results (Xu, L., et al., 2016). However, in this HT-HCA method, ND70 is used to label macropinosomes that range in size from 200 to 2,500 nm in diameter (Hewlett, L. J., et al., 1994; Kerr, M. C. and Teasdale, R. D., 2009). The large size of macropinosomes therefore obviates a need for high optical resolution.

The experiment utilizes phage-displayed, single-chain variable fragment (scFv) antibody libraries as a source for novel antibody clones. Multiple methods exist in generating phage-displayed antibody libraries (Andris-Widhopf, J., et al., 2011; Marks, J. D., et al., 1991; O'Connell, D., et al., 2002; Sheets, M. D., et al., 1998; Weber, M., et al., 2014). Phage display libraries need to be pre-selected against target tissues or cell types of interest to generate polyclonal outputs that are greatly enriched for binding clones; otherwise, the frequency of binding clones in the non-selected libraries is too low allow HT-HCA screening (Ha, K. D., et al., 2014). Multiple methods have been published that describe ways to select for high-affinity phage antibodies from cell and tissue specimen. For cell-based selection, phage antibody display libraries are incubated with live cells to enrich both surface-bound and internalized antibodies (An, F., et al., 2008; Liu, B., et al., 2004; Poul, M. A., et al., 2000; Zhu, X., et al., 2010). For tissue-based selection, we previously developed a novel method that involves selecting phage antibody libraries on cancer tissues with the aid of laser capture microdissection to specifically identify phage antibody binding to tumor cells in situ residing in their tissue microenvironment (Ruan, W., et al., 2006). Antibodies identified by this method bind to clinically relevant tumor epitopes and show excellent tumor targeting in vivo (He, J., et al., 2010; Iyer, A. K., et al., 2011; Iyer, A. K., et al., 2011; McCabe, K. E., et al., 2012). Alternatively, directed ultraviolet light can introduce genomic

cross-linking in phage antibody clones bound to undesired regions of tissues, thus rendering them non-replicable (Larsen, S. A., et al., 2015). Another method involves formalin-fixed, paraffin-embedded tissue biopsies on glass slides (Ten Haaf, A., et al., 2015).

The HT-HCA method begins with polyclonal phage-displayed antibody library selection outputs enriched for binding to target tissues or cell lines of interest. Colonies from the polyclonal selection outputs are picked and arrayed into 96-well plates for monoclonal culturing. Monoclonal phage antibodies are incubated with the cell line of interest and analyzed for binding by FACS. Positive binders are then re-arrayed and tested using HCA to further select for phage-antibodies that colocalize with ND70-TR (Ha, K. D., et al., 2014). Co-localization is quantified by PCC (or MOC) score, and clones with high PCC (or MOC) scores are further characterized by fluorescence confocal microscopy to quantify colocalization with the lysosomal marker lysosomal-associated membrane protein 1 (LAMP1). Clones that exhibit colocalization are then converted into full-length human antibodies, re-tested to confirm for both high-affinity binding and macropinocytosis-specific endocytosis, and then used to identify the corresponding receptor bound by the antibody. The method is summarized in a flow chart, depicted in Figure 1 (Ha, K. D., et al., 2014).

3. HT-HCA Screening Protocol for Macropinocytic Phage Antibodies

3.1. Monoclonal phage antibody array from a polyclonal selection output

1. Inoculate 1-5 mL of appropriate bacterial culture media with frozen glycerol stock of bacteria previously infected with the pre-selected phage antibody display library, containing appropriate antibiotic(s) (e.g. TG1 bacteria infected with fd phage displaying scFv antibody fragments are grown in 2X YT media containing 12.5 μg/mL tetracycline).

2. Measure A_{600}, where 1 O.D. is equivalent to about 6.8×10^8 cells/mL.

3. Dilute bacterial culture to yield ~90 cells/μL.

4. Plate 200-5,000 cells onto 10 cm agar-media plates containing the appropriate antibiotic(s). Plated cell counts will depend on bacterial cell viability within the glycerol stock.

5. Incubate plate(s) at 37 °C for 18 h.

6. After 18 h, the incubated plates should yield physically visible colonies with respectable spacing in between colonies to permit facile plucking using pipette tips or sterilized toothpicks.

7. Add 100-150 μL of bacterial culture media with appropriate antibiotic(s) into each well of a sterile, round-bottom, polystyrene or polypropylene 96-well plate with lid (BD Biosciences).

8. Inoculate each well with individual colonies using sterile pipette tips or toothpicks, leaving at least one well mock inoculated with a bacteria-free pipette tip or toothpick. This well will serve as a negative control to test for potential contamination.

9. Cover each 96-well plate with its accompanied lid and then cover each plate with aluminum foil.

10. Incubate 96-well plate(s) at 37 °C with shaking at 150-225 RPM for 18 h.

11. After 18 h, transfer 50 μL of bacterial/phage culture with a multi-channel pipette into wells of a new, sterile 96-well round-bottom plate.

12. Add 50 μL of bacterial media containing 50% glycerol to each well and pipette up/down five times to mix. Label and freeze plate at −80 °C as a glycerol stock plate.

13. The remaining 50-100 μL of bacterial/phage culture can be sealed with paraffin wax tape and stored at 4 °C for up to 24 h, to be used in high-affinity phage clone selection with tissue culture cells.

3.2. Identification of binding phage antibodies

1. Seed cell line of interest into sterile, optical flat-bottom 96-well plates with lids (BD Biosciences) to yield 70-90% confluence the next day.

2. The next day, centrifuge bacterial/phage culture 96-well plate at 3,900 g for 5 min and add 20 μL of supernatant containing phage particles into the respective wells of the 96-well plate containing tissue culture cells.

3. Incubate phage particles over tissue culture cells at 37 °C for 90 min, with standard carbon dioxide (CO_2, 5%) and humidity requirements for the cell line of interest.

4. After 90 min, gently wash unbound phage particles away from tissue culture cells using 150 μL of 1X phosphate buffered saline (PBS) twice.

5. Fix cells and any bound phages using 4% paraformaldehyde in PBS for 10 min at room temperature (RT).

6. Wash 3X with 150 μL of PBS.

7. Permeabilize cells with 150 μL of PBS containing 1% fraction V bovine serum albumin (BSA, Fisher Scientific) and 0.1% TritonX-100 (Sigma) for 15 min at RT.

8. Add 50 μL of biotinylated rabbit anti-bacteriophage fd antibody (Sigma, diluted 1:1,000 in PBS) to each well and incubate at RT for 1-2 h.

9. Wash twice with PBS.

10. Incubate wells with 50 μL of fluorophore-conjugated secondary antibody (e.g., phycoerythrin-conjugated streptavidin (Life Technologies/Invitrogen) or Alexa Fluor® 488-conjugated streptavidin (Jackson ImmunoResearch), final concentration1 μg/mL) in PBS buffer containing Hoechst 33342 dye at 1 μg/mL for 15-30 min at RT. Note: If the anti-bacteriophage antibody from 3.2.7 is already conjugated with a fluorophore, incubate cells with just Hoechst dye in PBS.

11. Wash wells thrice with PBS.

12. Image 96-well plates on automated, fluorescent plate imager such as CellInsight™ NXT HCS (Thermo Scientific), imaging a minimum of 300 cells per well. Cell count can be determined through Hoechst dye fluorescence.

13. For each phage-displayed antibody clone, clones yielding high anti-bacteriophage fluorescence values per cell are identified (typically 2 standard deviations above the mean).

14. Consolidate binding phage clones from their respective glycerol stock plates into fresh, sterile 96-well round-bottom plates, using 100-150 μL of bacterial media with appropriate antibiotic(s) (e.g., for fd phage, 2X YT with 12.5 μg/mL tetracycline).

15. Culture 96-well plates with at 37 °C with 150-225 RPM shaking for 18 h.

16. After 18 h, prepare glycerol stocks of the consolidated phage clones as described in 3.1.12, storing the remaining cultures at 4 °C for up to 24 h until further testing with HT-HCA.

3.3. HT-HCA screening of macropinocytosing phage antibodies

1. Seed tissue culture cell line of interest into flat-bottom 96-well plates with lids to yield 70-90% confluence the next day.

2. The next day, aspirate media from all wells and add 100 μL of media containing 70 kDa neutral dextran conjugated with Texas Red (ND70-TR, ThermoFisher/Life Technologies/Invitrogen) at a final concentration of 50 μg/mL.

3. Add 20 μL of supernatant from 3.2.16 to each well. Note: at this dilution, most of the tissue culture cell lines are viable for at least 24 h. If however, viability is affected by phage-containing supernatant, reduce the amount added (e.g., 10 or 5 μL) or shorten the time of incubation.

4. Incubate at 37 °C for 18 h, with standard carbon dioxide and humidity requirements for the tissue culture cell line of interest. Note: incubation time can vary between 8-18 h depending on the cell line.

5. After 18 h, gently wash unbound phage particles and non-internalized ND70-TR away from tissue culture cells using 150 μL of PBS twice.

6. Fix cells and any bound phages using 4% paraformaldehyde in PBS for 10 min at RT.

7. Wash with 150 μL of PBS.

8. Permeabilize cells for 15 min at RT.

9. Wash once with PBS, add 50 μL of biotinylated rabbit anti-bacteriophage fd antibody (Sigma, diluted 1:1,000 in 1:5 PBS-diluted permeabilization buffer) to each well and incubate at RT for 1-2 h.

10. Wash wells twice with PBS.

11. Incubate wells with 50 μL of fluorophore-conjugated secondary antibody (e.g., phycoerythrin-conjuagted streptavidin (Life Technologies/Invitrogen) or Alexa Fluor® 488-conjugated streptavidin (Jackson ImmunoResearch), final concentration1 μg/mL) in PBS buffer containing Hoechst 33342 dye at 1 μg/mL for 15-30 min at RT. Note: If the anti-bacteriophage antibody from 3.3.10 is already conjugated with a fluorophore, incubate cells with just Hoechst dye in 1:5 diluted permeabilization buffer.

12. Wash wells 3X with PBS.

13. Image the processed 96-well plate within an automated, fluorescent plate imager, e,g. CellInsight™ NXT HCS platform (Thermo Scientific) with a semi-aprochromat 20X LUCPLFLN objective (Olympus) utilizing >6 fields per well with a minimum of 300 cells per well.

14. Quantify colocalization through PCC (or MOC) score using analysis software associated with the instrument, utilizing Hoechst nuclei staining as the reference channel for cell counts (for CellInsight, use the built-in colocalization analysis). MOC analysis will require careful adjustment of fluorescent threshold settings and this threshold should be applied across all image fields. We typically use PCC in our HCA screening.

15. Phage antibody clones yielding high PCC values (typically 2 standard deviations above the mean of control cells) are identified as candidates with macropinocytosis-specific internalization qualities. Control cells should have bound phage antibodies previously identified that show no internalizing activity.

3.4. Sequencing, purification, and secondary characterization of phage clones

1. Culture bacteria/phage clones exhibiting ND70-TR colocalization in appropriate bacterial media with antibiotic(s).

2. Extract plasmid DNA from bacterial cultures and sequence.

3. Culture phage with unique antibody sequence in 100 mL bacterial culture media with appropriate antibiotic(s) for 18 h.

4. After 18 h, centrifuge bacteria at 3,900 g for 20 min at 4 °C.

5. Collect ~95 mL supernatant and filter-sterilize through 0.45 μm filters into sterile, conical tubes.

6. Add 25 mL 20% polyethylene glycol (PEG)-8000 (Sigma-Aldrich or VWR) in 2.5 M NaCl, mix, and incubate over ice for 5 h.

7. Centrifuge solution at 3,900 g for 20 min.

8. Carefully decant supernatant.

9. Resuspend the phage pellet in 10 mL PBS.

10. Add 2.5 mL 20% PEG-8000 in 2.5 M NaCl, mix, and incubate over ice for 10 min.

11. Centrifuge at 3,900 g for 20 min.

12. Carefully aspirate supernatant.

13. Resuspend pellets with 3 mL PBS, filter-sterilize through 0.45 μm filters into cryo-vials, and store at −80 °C. Note: alternatively, purified phages may be stored at 4 °C for many months, depending on the specific clone. Binding against tissue culture cells of interest will need to be re-assessed on a regular basis to determine stability and display level of purified phage stored at 4 °C.

14. Seed tissue culture cell line of interest onto a sterile 8-well Lab-Tek II chambered coverglass for subsequent fluorescent confocal microscopy and incubate at 37 °C for 18 h. Aim for 50-60% confluence by the next day.

15. Next day, incubate cells with complete tissue culture media containing ND70-TR at final 50 μg/mL and purified phage (1:10 dilution) for desired time period (e.g., 1 h, 4 h, 8 h, etc.).

16. Wash twice with PBS unbound phage particles and ND70-TR away from tissue culture cells.

17. Fix cells using 4% paraformaldehyde in PBS for 10 min at RT.

18. Wash twice with PBS.

19. Permeabilize cells with permeabilization buffer comprising PBS, 1% BSA and 0.1% TritonX-100, for 15 min at RT.

20. Add biotinylated rabbit anti bacteriophage fd antibody diluted at 1:1,000 in 1:5 PBS-diluted permeabilization buffer for 1 h at RT.

21. Wash twice with PBS.

22. Add to chamber fluorophore-conjugated secondary antibody (e.g., phycoerythrin-conjugated streptavidin (ThermoFisher/Life Technologies/Invitrogen) or Alexa Fluor® 488-conjugated streptavidin (Jackson ImmunoResearch), both at final concentrations of 1 μg/mL) in PBS buffer containing Hoechst 33342 dye at 1 μg/mL for 15-30 min at RT.

23. Wash 3X with PBS.

24. Image on a confocal microscope such as Fluoview (Olympus), capturing slices of cells that are sufficient to cover the entire Z-depth of each cell.

25. Quantify the degree of colocalization using on-board analysis software between phage and ND70-TR using PCC (using confocal Z-slices) or MOC (using Z-projected confocal images). Phage antibody clones yielding at least two standard deviations greater PCC or MOC values when compared to control phage antibody clones are considered candidates with macropinocytosis-specific internalization qualities. Standard deviation thresholds can be adjusted accordingly to limit or increase the number of potential, screened phage antibody clones. The control phage antibody should bind to the plasma membrane and does not internalize into the cell.

3.5. Converting single chain antibody fragments into human IgG antibodies

Phage antibodies that exhibit binding to tissue culture cells of interest and ND70-TR colocalization by HCA and confocal microscopy can then be converted into full-length human antibody for further analysis of internalization pathway and functional delivery.

1. Inoculate phage-infected TG1 bacteria in 3 mL of bacterial culture media with antibiotic(s) (e.g., 2X YT containing 12.5 μg/mL tetracycline) and incubate at 37 °C at 150-225 RPM for 18 h.

2. After 18 h, pellet bacteria at 8,000 *g* for 5 min.

3. Extract plasmid DNA from bacterial pellet using standard molecular biology techniques.

4. PCR-amplify heavy- and light-chain variable fragments separately from plasmid using appropriate primer sets (matching antibody sequences) and clone into either in-house expression vectors (Smith, K., et al., 2009) or commercial heavy and light chain expression vectors such as pFUSE-CHIg-hG1 for human IgG1 heavy chain (InvivoGen) and pFUSE2-CLIg-hK (InvivoGen) for human kappa light chain or pFUSE2-CLIg-hL2 (InvivoGen) for human lambda 2 light chain.

5. Prepare plasmid DNA of both the heavy and light expression constructs and sequence to confirm successful cloning.

3.6. Transient expression of full-length human IgGs

Full-length human IgG production is accomplished by transient co-transfection of a mix of heavy and light chain expression plasmids into HEK293a cells. For untested antibody clones, conduct small-volume, initial transfections before scaling up. The ratio of heavy to light can be varied to achieve the best results (we typically start with heavy to light at molar ratio 1:1). Stable cell line can be generated by electroporation of CHO cells, which is not covered by this protocol.

1. Seed HEK293a (ThermoFisher/Life Technologies/Invitrogen) into sterile 10 cm tissue culture plates or T-75 flasks to yield 80-90% confluence the next day.

2. Transfect cells using polyethylenimine (PEI) (Sigma-Aldrich) in Opti-MEM (ThermoFisher/Life Technologies/Invitrogen) or other lipid-based transfection means (e.g., Lipofectamine®3000 (ThermoFisher/Life Technologies/Invitrogen), using 10 μg of each heavy- and light-chain DNA construct for each 10 cm dish, mix according to the protocol of the transfection reagent.

3. For PEI-based transfection, incubate overnight at 37 °C with 5% CO_2, aspirate the transfection complexes and media from the tissue culture vessels and replace with serum-free media (50% DMEM/50% RPMI1640) supplemented with penicillin-streptomycin and Nutridoma-SP (Roche).

4. Collect after six days antibody-containing media from tissue culture vessels. Add fresh serum-free media over tissue culture vessels and repeat media collection three days later.

5. Centrifuge media at 5,000 g for 5 min at 4 °C using a bench top centrifuge and transfer supernatant into a fresh, sterile vessel. If available, quantify total IgG content within the collected media using a BLItz® system or equivalent label free interferometry method.

6. Store antibody-containing media at 4 °C for up to three days for future purification.

7. Before purification, FACS can be performed to confirm that the antibody can bind to the intended target cell line using procedures described below.

8. Detach target tissue culture cells from growth flasks or dishes using EDTA-based cell dissociation solution for 5-10 minutes at 37 °C.

9. Centrifuge detached cells at 700 g for 3 min and then aspirate the supernatant.

10. Incubate cells with supernatant containing human antibodies at RT for 1 h. Aim for 10 μg/mL antibody based on the BLItz® measurement. Note: if antibody concentrations in unpurified supernatant fall under 10 μg/mL but above 1 μg/mL, incubate cells with undiluted media. If less than 1 μg/mL antibody is present in the unpurified supernatant, the transfection may need to be repeated with varying heavy to light ratio. Antibodies that are poorly produced despite multiple trials are generally not moved to the next stage of analysis.

11. Wash cells twice with PBS/0.5% BSA, and incubate cells with secondary antibody (anti-human Fc specific antibody conjugated with a fluorophore)

12. Wash cells twice with PBS/0.5% BSA, and analyze binding by FACS.

3.7. Purification of full-length human IgG antibodies

This section largely adheres to standard, affinity-based antibody purification by protein A or G chromatography.

1. Prepare vertical drip column containing Protein A/G agarose slurry, utilizing the appropriate amount of slurry for the expected, total quantity of antibodies present in the media.

2. Wash columns with five bed volumes of PBS.

3. Add antibody-containing media to column of agarose beads and permit to drip through. Save the flow-through, stored on ice.

4. Wash columns thrice with five bed volumes of PBS.

5. Elute antibodies using 1X bed volume of 0.1 M glycine, pH 2.8.

6. Immediately neutralize with 1 M Tris-HCl, pH 8.0. Repeat elution and neutralization.

7. Quantify IgG in eluate fractions, and pool main antibody-containing fractions based on BLItz® measurement or equivalent label free interferometry method.

8. Concentrate pooled fractions using an Amicon® Ultra-4 centrifuge filter with 30,000 Dalton molecular weight cutoff (MilliPore) per manufacturer's recommendation. Buffer exchange is also accomplished at this step using either PBS or HEPES (pH 7.0).

9. Analyze purified IgG by 10% reducing SDS-PAGE. Note: we also perform HPLC (Agilent Technologies, 1220 infinity LC) analysis to determine aggregation status using the size exclusion column. IgGs that show more than 10% aggregation are not moved to the next stage of analysis as internalization is sensitive to crosslinking by aggregated antibodies. Those aggregation prone antibodies may be "rescued" by mutagenesis or chain shuffling, which is beyond the scope of this protocol.

3.8. Confirming human IgG antibody internalization into cells through macropinocytosis

It is important to confirm that the full-length human antibody is indeed internalized by macropinocytosis as indicated by the phage antibody because (1) internalization pathway could be different between single chain and full-length antibodies, and (2) phage antibody can non-specifically interact with cell membrane to yield internalization patterns/properties that are not truly associated with the target receptor.

1. Seed tissue culture cell line of interest onto a sterile 8-well Lab-Tek II chambered coverglass and aim for 50-60% confluence by the next day.

2. Next day, incubate cells with tissue culture media containing ND70-TR (final concentration at 50 μg/mL) and purified IgG (final concentration at 10 μg/mL) for various time points.

3. Wash cells twice with PBS to remove any unbound IgG antibodies and ND70-TR.

4. Fix cells using 4% paraformaldehyde in PBS for 10 min at RT.

5. Wash 3X with PBS.

6. Permeabilize cells with permeabilization buffer comprising PBS, 1% BSA and 0.1% TritonX-100, for 15 min at RT.

7. Incubate cells with antibodies against various organelles such as lysosomes (LAMP1) and early endosomes (early endosomal antigen 1 or EEA1).

8. Wash 3X with PBS.

9. Incubate cells with fluorescently-conjugated anti-human Fc secondary antibody and fluorescently-conjugated secondary antibodies against anti-organelle antibodies, diluted in 1:5 PBS-diluted permeabilization buffer and Hoechst dye for 30 min.

10. Wash twice with PBS, and fix cells with 4% paraformaldehyde in PBS for 10 min RT.

11. Image on confocal microscope as described in 3.4.24.

12. Quantify the degree of colocalization between IgG antibody and ND70-TR (and organelles) using either PCC (using confocal Z-slices) or MOC (using Z-projected confocal images).

3.9. Identification of target receptor bound by macropinocytosing antibody

Once a macropinocytosing antibody is identified and characterized, it can be used to identify the bound antigen. This procedure depends on antibody affinity capture of membrane-bound receptors that are labeled by cell surface biotinylation (Goenaga, A. L., et al., 2007; Liu, B., et al., 2007; Ruan, W., et al., 2006). Following immunoprecipitation of cell lysates using the target antibody of interest and a control antibody binding to a known antigen against the same cell line, the immunoprecipitate is analyzed by SDS-PAGE and immunoblot that reveal protein bands unique to the immunoprecipitate pulled down by antibody of interest (Ha, K. D., et al., 2014). The corresponding protein bands can then be excised from an SDS-PAGE, and analyzed by tandem mass spectrometry (Andersen, J. D., et al., 2010; Liu, B., et al., 2002; Lund, T. C., et al., 2007). Other novel methods for antigen identification, such as screening yeast surface cDNA display libraries, can also be used to identify antigens bound by either phage or full-length antibodies (Bidlingmaier, S., et al., 2009; Bidlingmaier, S. and Liu, B., 2006). These methods are described in details elsewhere (Bidlingmaier, S. and Liu, B., 2011; Bidlingmaier, S., et al., 2015) and are beyond the scope of this chapter.

1. Seed target tissue culture cells of interest to yield roughly 9×10^6 cells the next day for each antibody (target and control antibodies).

2. The next day, harvest and pellet cells at 1,000 g for 5 min at 4 °C.

3. Wash cell pellet 3X with PBS (pH 8.0), centrifuging cells at 1,000 g for 5 min after each wash.

4. Resuspend cells at a concentration of 2.5×10^7 cells/mL with PBS (pH 8.0).

5. Equilibrate EZ-link sulfo-NHS-LC-biotin (Thermo Pierce #21335) to RT prior to opening the reagent vessel.

6. Prepare 10 mM biotin labeling reagent in PBS.

7. Immediately add 200 μL of reagent per mL of resuspended cells and incubate on ice for 20 min with occasional agitation.

8. Wash cell pellet 3X with PBS (pH 8.0) containing 100 mM glycine to quench the reaction.

9. 116. Lyse cell pellet with lysis buffer, such as RIPA lysis buffer (50 mM Tris-HCl, pH 7–8, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40).

10. Centrifuge lysates in a refrigerated microcentrifuge (Eppendorf) at 14,000 g at 4 °C for 10 min.

11. Collect supernatant and incubate with 150 μL of protein A agarose (ThermoFisher/Pierce) slurry per mL of lysate.

12. Pre-clear lysate by incubating with protein A agarose slurry overnight at 4 °C with gentle rotation or at RT for 3 hours with gentle rotation.

13. Spin down beads at 5,000 g for 1 min and collect pre-cleared, biotinylated lysates.

14. Store at −80 °C or use immediately for immunoprecipitation.

15. Conjugate target antibody onto protein A/G agarose beads. Additionally conjugate a separate control antibody that binds to a known antigen expressed on the cell line of interest. This can be performed with chemical crosslinking by DMP (Sigma #D8388), as described in the next steps.

16. Bind roughly 2 mg of antibody per mL of wet protein A/G agarose beads in PBS (pH 7.4), incubate for 1 h at RT with gentle rotation, or 4 °C overnight.

17. Wash beads twice with 10 bead volumes of 0.2 M sodium borate (pH 9.0), centrifuging beads at 1,000 g for 1 min at 4 °C.

18. Resuspend beads in 10 bead volumes of 0.2 M sodium borate (pH 9.0).

19. Remove roughly 10 μL of agarose beads for future SDS-PAGE analysis (as pre-conjugated antibody-beads sample).

20. Add DMP (Sigma #D8388) to bead slurry to yield a final DMP concentration of 13 mg/mL.

21. Incubate for 30 min at RT with gentle rotation.

22. Stop the reaction by pelleting and washing beads with 10 bed volumes of 0.2 M ethanolamine in PBS (pH 8.0).

23. Incubate beads with ethanolamine solution for 2 h at RT with gentle rotation.

24. Pellet beads and resuspend with PBS (pH 7.4) containing 0.02% sodium azide.

25. Remove 10 μL of agarose beads, for future SDS-PAGE analysis (as post-conjugated antibody-beads sample).

26. Check the efficiency of antibody conjugation onto agarose beads by performing SDS-PAGE on the pre- and post-conjugated antibody-beads samples. Efficient conjugation is indicated by heavy chain presence (~55 kDa) in the pre-conjugation sample, but not in the post-conjugation sample.

27. Add pre-cleared biotinylated cell lysates to target antibody-conjugated agarose beads and incubate at RT with gentle rotation for 1 h or 4 °C overnight. Additionally, perform the same for the control antibody.

28. Pellet beads at 14,000 g for 5 min at 4 °C using a refrigerated microcentrifuge.

29. Aspirate remaining supernatant and wash beads 3X with 10 bed volumes of lysis buffer used, such as the RIPA buffer.

30. Split slurry in half into two separate tubes, one will be used for SDS-PAGE and the other for immunoblotting with horseradish peroxidase (HRP)-conjugated streptavidin.

31. Add appropriate amount of 5X SDS sample buffer to pelleted beads and boil beads for 2 min at >95 °C.

32. Run two SDS-PAGE gels in parallel, where one gel is stained with GelCode (ThermoFisher), or other mass spectrometry-safe colloidal stain, and the other gel is Western-transferred to PVDF membrane and immunoblotted with streptavidin-HRP.

33. The band(s) on the immunoblot that is unique to the target antibody, but not the control antibody, indicates the gel position(s) of the receptor(s) bound by the antibody.

34. Excise the corresponding protein band(s) from the GelCode-stained gel and then submit the excised gel fragment(s) for protein identification through tandem mass spectrometry. In-gel trypsin digestion, sample analysis by tandem mass spectrometry (MS/MS), and analysis of MS/MS data using Sequest (ThermoFisher) are commonly available (e.g., http://cbs.umn.edu/cmsp/home).

35. Confirm antigen identification by ectopic expression of the cDNA encoding the putative target antigen in CHO or HEK293 cells that do not express the target (not bound by the antibody), followed by FACS analysis (Ha, K. D., et al., 2014). Alternatively, if antibodies against the putative target antigen are available commercially, they can be used in Western blot analysis to confirm the target antigen identification (Ruan, W., et al., 2006).

3.10. Functional internalization and targeted payload delivery

Besides microscopic studies, a functional internalization assay can be used to confirm antibody internalization and demonstrate cell type-specific intracellular payload delivery. The assay is based on saporin (Stirpe, F., et al., 1983), a plant toxin that is not internalized on its own but is internalized when conjugated to an internalizing antibody. Internalization of saporin inhibits protein synthesis of within the target cell, resulting in cell death (Rahman, W., et al., 2008; Vago, R., et al., 2005). The following procedure is based on biotin-labeling the macropinocytosing antibody, mixing with streptavidin-conjugated toxin to form the immunotoxin, and incubating the immunotoxin with target and control cells to assess cell-type specific loss of viability.

1. To biotin-label antibody, remove the vial of Sulfo-NHS-LC-Biotin from −20 °C and warm it to RT before use.

2. Immediately before use, prepare a 10 mM EZ-Link Sulfo-NHS-LC-Biotin solution using ultrapure water.

3. Add 27 μL of 10 mM biotin solution to 1 mL of 2 mg/mL purified human IgG antibody in PBS at pH 7.4, rotate the mixture at RT for 45 min.

4. Add 20% v/v of 1 M Tris-HCl (pH 8.0) to quench the reaction, mix gently by pipetting up and down.

5. Buffer-exchange to PBS and remove nonreacted biotin using Zeba™ spin desalting columns (Thermo fisher) according to the manufacturer's instruction. Biotin-labeled antibody can be stored at −20 °C for months or 4 °C for a couple of weeks until use in immunotoxin assays.

6. Seed target and control cell lines (e.g., tumor vs. non-tumor lines) at a density of 1,000-2,000 cell per well in a 96-well flat bottom plates with 50 μL growth media and grow for 16 h at 37 °C with 5% CO_2.

7. Mix streptavidin-conjugated saporin (SA-ZAP, Advanced Targeting Systems) with biotinylated IgG at a molar ratio of 1:1, vortex and incubate on ice for 30 min to form the immunotoxin.

8. Add 50 μL serially diluted immunotoxin in PBS to each well and incubate for 96 h at 37 °C with 5% CO_2. For initial assessment, 1:10 serial dilution is often use to find the range. For assessment of the half maximal effective concentration (EC_{50}), 1:3 serial dilution is often used to improve accuracy.

9. Remove the cell growth media from each well, add 100 μL of diluted CCK-8 solution (Dojindo, 10 μL CCK-8 solution mixed with 90 μl PBS) to each well in the 96-well plates, and incubate for 1-4 h at 37 °C in 5% CO_2. Note: remove any air bubble in the well, as it interferes with absorbance reading.

10. Measure the absorbance at 450 nm using a microtiter plate reader (e.g., Synergy HT from Bioteck).

11. Determine the EC_{50} value by curve fitting using Prism (GraphPad).

4. Conclusion

The method described enables identification of macropinocytosis-specific internalizing antibodies through a high content analysis-based screening of phage antibody display libraries. Novel phage antibodies are identified by co-localization with macropinocytosis marker, converted into full length human antibodies and further characterized with regard to cell binding, pathway of internalization, and intracellular payload delivery. Target antigen can be identified by immunoprecipitation and mass spectrometry analysis. This HT-HCA screening platform is generally applicable and should allow systematic discovery, evaluation and exploitation of macropinocytosis, a subset of the internalizing space, whose activity is significantly upregulated in many types of cancer.

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References

Adler J, Parmryd I, (2010). Quantifying colocalization by correlation: the Pearson correlation coefficient is superior to the Mander's overlap coefficient, Cytometry A 77(8) 733–42. [PubMed: 20653013]

An F, et al. , (2008). Targeted drug delivery to mesothelioma cells using functionally selected internalizing human single-chain antibodies, Mol Cancer Ther 7(3) 569–78. [PubMed: 18319332]

Andersen JD, et al. , (2010). Identification of candidate biomarkers in ovarian cancer serum by depletion of highly abundant proteins and differential in-gel electrophoresis, Electrophoresis 31(4) 599–610. [PubMed: 20162585]

Andris-Widhopf J, et al. , (2011). Generation of human scFv antibody libraries: PCR amplification and assembly of light- and heavy-chain coding sequences, Cold Spring Harb Protoc 2011(9).

Behrens CR, Liu B, (2014). Methods for site-specific drug conjugation to antibodies, MAbs 6(1) 46–53. [PubMed: 24135651]

Bidlingmaier S, et al. , (2009). Identification of MCAM/CD146 as the target antigen of a human monoclonal antibody that recognizes both epithelioid and sarcomatoid types of mesothelioma, Cancer Res 69(4) 1570–7. [PubMed: 19221091]

Bidlingmaier S, Liu B, (2006). Construction and application of a yeast surface-displayed human cDNA library to identify post-translational modification-dependent protein-protein interactions, Mol Cell Proteomics 5(3) 533–40. [PubMed: 16321969]

Bidlingmaier S, Liu B, (2011). Identification of protein/target molecule interactions using yeast surface-displayed cDNA libraries, Methods Mol Biol 729 211–23. [PubMed: 21365493]

Bidlingmaier S, et al. , (2015). Combining Phage and Yeast Cell Surface Antibody Display to Identify Novel Cell Type-Selective Internalizing Human Monoclonal Antibodies, Methods Mol Biol 1319 51–63. [PubMed: 26060069]

Carter PJ, Senter PD, (2008). Antibody-drug conjugates for cancer therapy, Cancer J 14(3) 154–69. [PubMed: 18536555]

Commisso C, et al. , (2013). Macropinocytosis of protein is an amino acid supply route in Ras-transformed cells, Nature 497(7451) 633–7. [PubMed: 23665962]

Dunn KW, et al. , (2011). A practical guide to evaluating colocalization in biological microscopy, Am J Physiol Cell Physiol 300(4) C723–42. [PubMed: 21209361]

Goenaga AL, et al. , (2007). Identification and characterization of tumor antigens by using antibody phage display and intrabody strategies, Mol Immunol 44(15) 3777–88. [PubMed: 17498801]

Ha KD, et al. , (2014). High-content analysis of antibody phage-display library selection outputs identifies tumor selective macropinocytosis-dependent rapidly internalizing antibodies, Mol Cell Proteomics 13(12) 3320–31. [PubMed: 25149096]

He J, et al. , (2010). Targeting prostate cancer cells in vivo using a rapidly internalizing novel human single-chain antibody fragment, J Nucl Med 51(3) 427–32. [PubMed: 20150269]

Hewlett LJ, et al. , (1994). The coated pit and macropinocytic pathways serve distinct endosome populations, J Cell Biol 124(5) 689–703. [PubMed: 8120092]

Huang S, et al. , (2015). Comparison of Dextran Perfusion and GSI-B4 Isolectin Staining in a Mouse Model of Oxygen-induced Retinopathy, Eye Sci 30(2) 70–4. [PubMed: 26902065]

Iyer AK, et al. , (2011). Novel human single chain antibody fragments that are rapidly internalizing effectively target epithelioid and sarcomatoid mesotheliomas, Cancer Res 71(7) 2428–32. [PubMed: 21447742]

Iyer AK, et al. , (2011). The effect of internalizing human single chain antibody fragment on liposome targeting to epithelioid and sarcomatoid mesothelioma, Biomaterials 32(10) 2605–13. [PubMed: 21255833]

Kerr MC, Teasdale RD, (2009). Defining macropinocytosis, Traffic 10(4) 364–71. [PubMed: 19192253]

Larsen SA, et al. , (2015). Selection of cell-type specific antibodies on tissue-sections using phage display, J Cell Mol Med 19(8) 1939–48. [PubMed: 25808085]

Liu B, et al. , (2004). Mapping tumor epitope space by direct selection of single-chain Fv antibody libraries on prostate cancer cells, Cancer Res 64(2) 704–10. [PubMed: 14744788]

Liu B, et al. , (2007). Recombinant full-length human IgG1s targeting hormone-refractory prostate cancer, J Mol Med 85(10) 1113–23. [PubMed: 17554518]

Liu B, et al. , (2002). Towards proteome-wide production of monoclonal antibody by phage display, J Mol Biol 315(5) 1063–73. [PubMed: 11827476]

Lund TC, et al. , (2007). iTRAQ is a useful method to screen for membrane-bound proteins differentially expressed in human natural killer cell types, J Proteome Res 6(2) 644–53. [PubMed: 17269721]

Marks JD, et al. , (1991). By-passing immunization. Human antibodies from V-gene libraries displayed on phage, J Mol Biol 222(3) 581–97. [PubMed: 1748994]

McCabe KE, et al. , (2012). An engineered cysteine-modified diabody for imaging activated leukocyte cell adhesion molecule (ALCAM)-positive tumors, Mol Imaging Biol 14(3) 336–47. [PubMed: 21630083]

O'Connell D, et al. , (2002). Phage versus phagemid libraries for generation of human monoclonal antibodies, J Mol Biol 321(1) 49–56. [PubMed: 12139932]

Poul MA, et al. , (2000). Selection of tumor-specific internalizing human antibodies from phage libraries, J Mol Biol 301(5) 1149–61. [PubMed: 10966812]

Rahman W, et al. , (2008). Selective ablation of dorsal horn NK1 expressing cells reveals a modulation of spinal alpha2-adrenergic inhibition of dorsal horn neurones, Neuropharmacology 54(8) 1208–14. [PubMed: 18462764]

Ruan W, et al. , (2006). Identification of clinically significant tumor antigens by selecting phage antibody library on tumor cells in situ using laser capture microdissection, Mol Cell Proteomics 5(12) 2364–73. [PubMed: 16982673]

Scott AM, et al. , (2012). Antibody therapy of cancer, Nat Rev Cancer 12(4) 278–87. [PubMed: 22437872]

Sheets MD, et al. , (1998). Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens, Proc Natl Acad Sci U S A 95(11) 6157–62. [PubMed: 9600934]

Sherbenou DW, et al. , (2015). The development of potential antibody-based therapies for myeloma, Blood Rev 29(2) 81–91. [PubMed: 25294123]

Smith K, et al. , (2009). Rapid generation of fully human monoclonal antibodies specific to a vaccinating antigen, Nat Protoc 4(3) 372–84. [PubMed: 19247287]

Stirpe F, et al. , (1983). Ribosome-inactivating proteins from the seeds of Saponaria officinalis L. (soapwort), of Agrostemma githago L. (corn cockle) and of Asparagus officinalis L. (asparagus), and from the latex of Hura crepitans L. (sandbox tree), Biochem J 216(3) 617–25. [PubMed: 6667259]

Ten Haaf A, et al. , (2015). Phage display-based on-slide selection of tumor-specific antibodies on formalin-fixed paraffin-embedded human tissue biopsies, Immunol Lett 166(2) 65–78. [PubMed: 26045318]

Vago R, et al. , (2005). Saporin and ricin A chain follow different intracellular routes to enter the cytosol of intoxicated cells, FEBS J 272(19) 4983–95. [PubMed: 16176271]

Weber M, et al. , (2014). A highly functional synthetic phage display library containing over 40 billion human antibody clones, PLoS One 9(6) e100000. [PubMed: 24950200]

Xu L, et al. , (2016). Resolution, target density and labeling effects in colocalization studies - suppression of false positives by nanoscopy and modified algorithms, FEBS J 283(5) 882–98. [PubMed: 26756570]

Zhu X, et al. , (2010). Identification of internalizing human single-chain antibodies targeting brain tumor sphere cells, Mol Cancer Ther 9(7) 2131–41. [PubMed: 20587664]
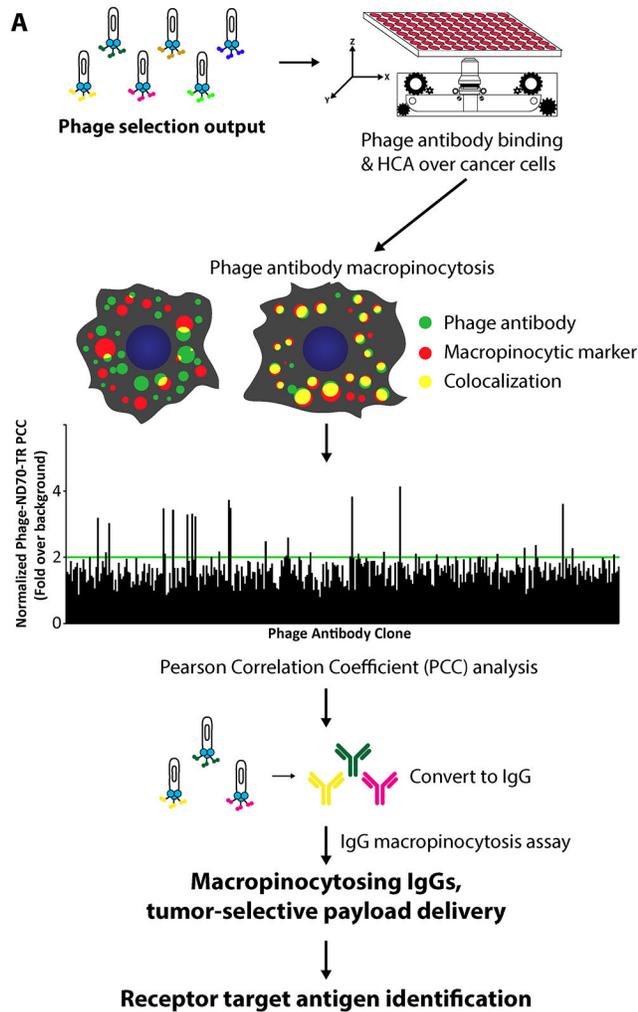
Figure 1:
Outline of HCA screening strategy for identification of macropinocytosing phage antibody. Sublibraries generated previously by us from laser capture microdissection-based phage antibody library selection (Ruan, W., et al., 2006) are used as the starting material for screening. HCA instruments enables automated high throughput detection of antibody colocalization with a macropinocytosis marker, which is quantified by PCC scores. Phage antibodies with PCC scores above two standard deviations of the mean are identified and converted to full-length human IgGs for further characterization, including macropinocytosis, tumor-selective payload delivery and target antigen identification. The figure is adopted from our original publication in Mol. Cell. Proteomics (Ha, K. D., et al., 2014).