Methods xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Detection and isolation of auto-reactive human antibodies from primary B cells

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ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Single-cell
Antibodies
Autoimmunity
Nanowell array
ACPA

ABSTRACT

The isolation of human monoclonal antibodies (hmAb) has emerged as a versatile platform in a wide variety of contexts ranging from vaccinology to therapeutics. In particular, the presence of high titers of circulating auto-antibodies is implicated in the pathology and outcome of autoimmune diseases. Therefore, the molecular characterization of these hmAb provides an avenue to understanding the pathogenesis of autoimmune diseases. Additionally, the phenotype of the auto-reactive B cells may have direct relevance for therapeutic intervention. In this report, we describe a high-throughput single-cell assay, microengraving, for the screening, characterization and isolation of anti-citrullinated protein antibodies (ACPA) from peripheral blood mononuclear cells (PBMC) of rheumatoid arthritis (RA) patients. Stimulated B cells are profiled at the single-cell level in a large array of sub-nanoliter nanowells ($\sim 10^5$), assessing both the phenotype of the cells and their ability to secrete cyclic-citrullinated peptide (CCP)-specific antibodies. Single B cells secreting ACPA are retrieved by automated micromanipulation, and amplification of the immunoglobulin (Ig) heavy and light chains is performed prior to recombinant expression. The methodology offers a simple, rapid and low-cost platform for isolation of auto-reactive antibodies from low numbers of input cells and can be easily adapted for isolation and characterization of auto-reactive antibodies in other autoimmune diseases.

1. Introduction

In the last couple of decades, the ability to isolate fully human monoclonal antibodies (hmAb) from B cells has emerged as a versatile platform for many applications including the production of therapeutic antibodies [1], revealing molecular insights into the nature of antigen driven antibody affinity maturation [2], structural vaccinology [3,4], recognition of conserved viral epitopes [5], and elucidating fundamental mechanisms of B cell immunology in autoimmune diseases [6].

Donor-derived hmAb are isolated by immortalization of primary B cells employing traditional methods like the hybridoma technology [7] or *in vitro* infection with Epstein–Barr virus [8], or by utilizing more recent methodologies like genetic reprogramming of memory B cells [9]. The advantage of these approaches is that upon immortalization the cells serve as production factories for the secretion of the native hmAb. The drawbacks however are that immortalization efficiencies are not high and the cells still need to be screened in a second step to isolate antigen-specific clones. Alternately, primary B cells can be directly interrogated for their antigen specificity using either flow-cytometry or microwell arrays [10–12], and single antigen-specific B cells can be isolated for reverse transcription, gene amplification, cloning and recombinant expression of the hmAb [13,14]. The advantages of these approaches are that they are easier to implement, rapid and facilitate screening up front. Secondly, with regards to the micro/nanowell arrays, the ability to work with small sample sizes like tissue resident B cells, and the ability to screen both memory B cells and antibody-secreting plasmablasts and plasma cells are added advantages. A limitation of these approaches, however is that they rely on recombinant antibody expression.

The isolation of auto-antibodies, antibodies directed against self-antigens, holds promise as a mechanism to delineate the molecular basis of autoimmune diseases [15]. Auto-antibodies that are highly specific for cellular antigens can be detected both in the sera and target organs of patients with organ-specific autoimmune diseases such as rheumatoid arthritis (RA), type I diabetes and thyroiditis [16]. In RA patients, the presence of these auto-antibodies like the anti-citrullinated protein antibodies (ACPA) has diagnostic and prognostic significance [17–19]. In line with other similar autoimmune diseases, it has also been demonstrated, that the ACPA may contribute to development of inflammatory arthritis

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http://dx.doi.org/10.1016/j.ymeth.2013.06.018

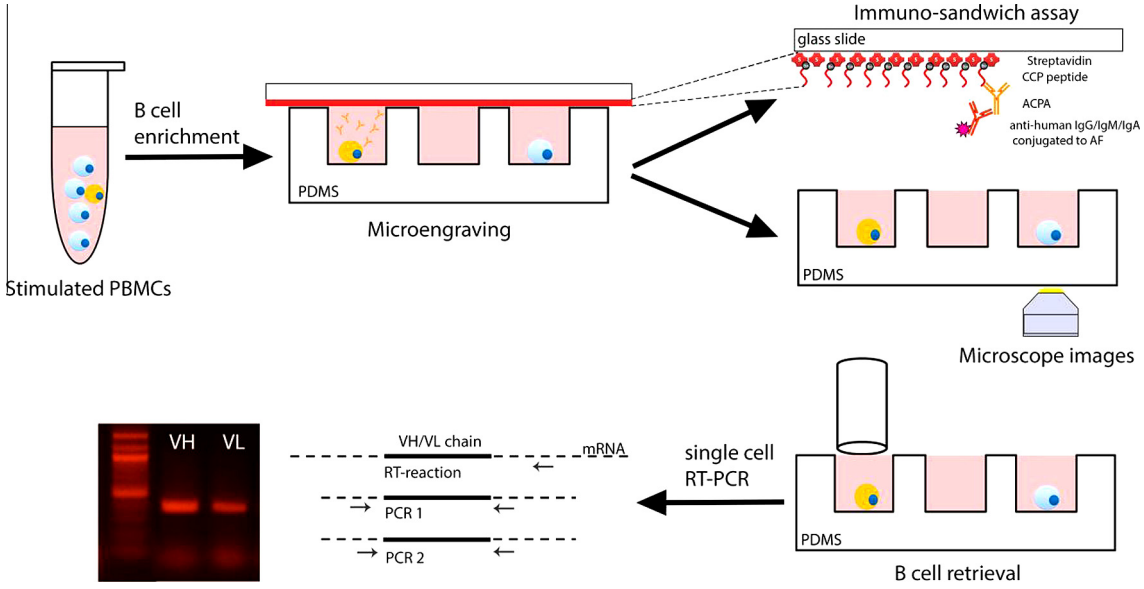

Fig. 1. Outline of the methodology for the combined screening of the antigen-specificity and phenotype of single auto-reactive B cells. B cells obtained by enrichment from CCP$^+$ donor derived PBMCs are loaded onto a nanowell array. Microengraving is used to determine CCP reactivity and automated fluorescence microscopy is employed to determine the phenotype of the B cells, on-chip. Post data-analysis, the memory B cells secreting CCP-specific hmAb are retrieved by micromanipulation. Single cell RT-PCR is performed to enable the amplification of the Ig V_H and V_L regions.

[20,21]. Consistent with this finding, therapeutic regimens that utilize antibody-mediated depletion of B cells in autoimmune diseases, may provide clinical benefit [22,23]. Thus, in addition to the molecular characterization of ACPA, determining the phenotype of auto-reactive B cells is essential for the development of clinical strategies that rely on B cell depletion [24,25].

Here, we describe a novel high-throughput technology, that allows for the combined screening of the phenotype and antigen-specificity of ACPA secreted from single B cells. In this approach, PBMC are briefly stimulated *ex vivo* with recombinant human interleukin-21 (rhIL-21) and soluble CD40 ligand (sCD40L) to facilitate the generation of antibody secreting cells (ASC), as described previously [26]. The enriched B cell population is then loaded onto a microfabricated nanowell array ($\sim 10^5$ individual nanowells per array) with sub-nanoliter volumes (125 pL) to isolate individual cells. The nanowell array is interrogated for cyclic-citrullinated peptide (CCP) [17] specific immunoglobulin (Ig) secretion by using a functionalized glass slide. In combination with automated fluorescence microscopy, CCP-specific live B cells are identified and retrieved by micromanipulation. Subsequently, single cell RT-PCR is performed to amplify Ig variable heavy and light chain (V_H:V_L) genes from the retrieved B cells. The results outline a workflow to obtain paired Ig V_H and V_L gene amplification by screening, identification and isolation of CCP-specific memory B cells from RA patients PBMC (Fig. 1). This methodology provides a fast, efficient and economical platform for isolation of antigen-specific antibodies.

The protocol described here, demonstrated in the context of ACPA, can be readily adapted to the screening of hmAb against any antigen of interest.

2. Methods

2.1. Human subjects statement

All work outlined in this report was performed according to protocols approved by the Institutional Review Boards at the University of Houston (12495-EX) and the Baylor College of Medicine (H30360).

2.2. Detection of antigen-specific antibodies

As outlined previously, the detection of antigen-specific hmAb from single B cells has been most commonly accomplished via the use of flow cytometry. Labeled antibodies directed against B-cell phenotypic markers like CD19 and CD20 are combined with screens for either soluble antigen or even whole cells displaying antigen [5,10]. The sorted single cells are then cloned for recombinant expression and assayed for their antigen specificity. Similarly, high-throughput cloning of single B cells has been employed to isolate panels of hmAb via recombinant expression, and the antigen specificity of these hmAb is determined in a second step using ELISA [6].

More recently, microfabricated arrays have been described for detection of antigen specificity of single B cells by either direct interrogation of surface-bound B cell receptor (BCR) [27] or Ca^{2+} mobilization [28], or by detecting the secreted hmAb (by antibody secreting cells (ASC) or stimulated B cells) [11]. We outline a protocol here for the detection of secreted hmAb by activated single B cells using microengraving.

2.2.1. Detection of ACPA in RA patient sera

A commercial ELISA-based test, QuantaLite CCP (Innova Diagnostic, San Diego, CA), is used in the clinic for the diagnosis of CCP$^+$ RA. We have adapted this ELISA to enable us to pre-screen RA patients with moderate to high-titers of ACPA (Fig. 2A). Briefly, streptavidin-coated microplates are used to capture biotinylated CCP and subsequently incubated with the plasma samples. Detection is accomplished using a secondary horseradish peroxidase (HRP)-conjugated antibody. In our example, since RA02 plasma has higher ACPA titers, we chose this sample for screening ACPA from activated B cells (Fig. 2A).

1. Prepare PBST by adding 0.05% Tween 20 (Sigma–Alrdich, St. Louis, MO) to 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4).
2. Coat 96-well polystyrene MaxiSorp plates (NUNC, Rochester, NY) with 100 μL/well of 50 μg/mL streptavidin (Sigma–Aldrich). Seal the plates with Parafilm M (Pechiney, Chicago, IL), and

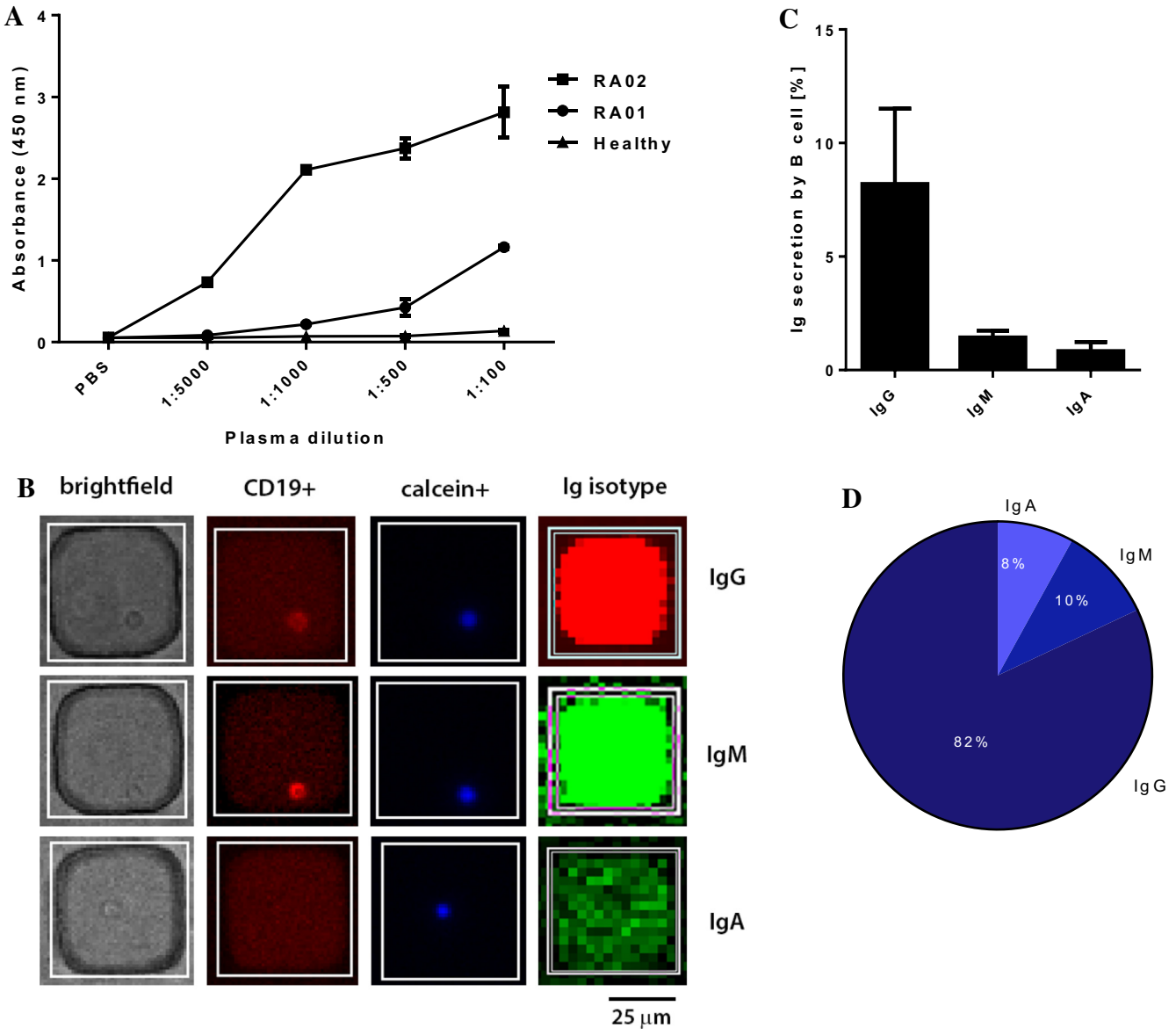

Fig. 2. Characterization of ACPA. (A) CCP reactivity of donor plasma as determined by ELISA. Briefly, microtiter plates are coated with streptavidin, incubated first with biotinylated-CCP and then with plasma at different dilutions. Detection is enabled by incubation with HRP conjugated to anti-human IgG/IgA, followed by colorimetric readout. (B) Micrographs of matched images of single B cells to the isotype of the antibody secreted, detected by microengraving. (C) The frequency of immunoglobulin (IgG, IgM and IgA) secretion from three independent sets of stimulated, enriched B cells isolated from the PBMC of RA$^-$ donors, determined by microengraving. (D) The distribution of immunoglobulin isotype of these same samples.

incubate overnight at 4 °C. Wash 5 times with PBST and block with at least 200 μL/well of 10% bovine serum albumin (BSA) in PBST.
3. Incubate at room temperature (RT) for 2 h.
4. Wash 5 times with PBST and incubate with 100 μL/well of 5 μg/mL biotinylated CCP (Anaspec, Fremont, CA). Seal the plates and incubate at RT for 1 h. Wash 5 times with PBST.
5. Add 100 μL/well of the human plasma samples to be tested, at the dilutions 1:100, 1:500, 1:1000, and 1:5000 (each in duplicate). Add 100 μL of 1× PBS as negative control. Seal the plates and incubate at RT for 45 min.
6. Wash 5 times with PBST. Add 100 μL/well of goat anti-human IgG/IgA HRP-conjugated antibody (Innova Diagnostic) and incubate for 1 h at RT. Wash 5 times with PBST.
7. Add 100 μL/well of TMB (3,3′,5,5′-tetramethylbenzidine) substrate (Thermo, Waltham, MA) and incubate at RT for 10 min until the blue color develops.
8. Quantify the absorbance at 450 nm using the InfiniteM300 Microplate Reader (Tecan, Männedorf, Switzerland).

2.3. Optimization of memory B cell stimulation

In order to convert B cells into antibody-secreting cells *in vitro*, a number of different protocols employing cytokines, TLR agonists, CD40L and antibodies have been reported [29–31]. Each of these methods induces secretion from slightly different subsets of B cells. In our experiments, the use of sCD40L, rhIL-21 and anti-APO1 enables secretion from the highest frequency of B cells using minimal culture times. sCD40L engages with CD40 expressed on the cell surface of B cells to mimic T cell-mediated activation [32] and IL-21 is known to promote the differentiation to antibody-secreting cells [26,33]. Since activation also induces cell death, anti-APO1 is used to rescue B cells from Fas-induced apoptosis [34].

We have used PBMC isolated by Ficoll-Paque density gradient centrifugation of fresh whole blood. After 4 days of stimulation, the frequency of antibody-secreting B cells is assessed by microengraving. The percentage of activated B cells is calculated as the number of live B cells secreting Ig as a fraction of the total number of CD19 positive, live B cells present in the nanowell array.

These results indicate that 6–13% of B cells secrete antibodies, with IgG as a predominant isotype, consistent with selective stimulation of memory B cells (Fig. 2C and D).

1. Prepare RPMI-PLGH media: 500 mL RPMI-1640 (Cellgro, Manassas, VA), Penicillin–streptomycin (Cellgro) 50,000 U/50 mg + L-glutamine 2 mM (Cellgro) + HEPES 10 mM (Sigma).
2. Prepare R10 media with RPMI-PLGH media supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (Biological, Lawrenceville, GA).
3. Thaw previously frozen PBMC in R10 media in presence of 20 μg/mL of DNAseI (Sigma). Wash twice with R10 and re-suspend to 3×10^6 cells/mL in R10 media.
4. Add the stimulation cocktail: 2.5 μg/ml sCD40L (R&D System, Minneapolis, MN), 50 ng/mL rhIL-21 (BD Bioscience, San Jose, CA), and 5 μg/mL anti-APO1 (eBioscience, San Diego, CA) and mix thoroughly.
5. Incubate at 37 °C, 5% CO_2 for 4 d.

2.3.1. B cell enrichment

B cell enrichment is performed in order to increase the number of B cells assayed on a single nanowell array. The protocol is performed according to manufacturer's instructions using the EasySep™ Human B Cell Enrichment kit (Stemcell Technologies, Vancouver, Canada). Briefly, 5×10^6 of PBMC are re-suspended in 100 μL of recommended media and placed in a round-bottom 96-well plate. Subsequent to the addition of 5 μL of the Human B Cell Enrichment Cocktail, the mixture is incubated for 10 min at RT and 10 μL of the D magnetic particles are added. After a further incubation for 5 min, 135 μL of recommended medium is added and plate is positioned onto the EasyPlate Magnet (Stemcell Technologies) for 10 min. The negatively selected, enriched B cells are carefully removed and transferred to a separate well until use.

2.4. Screening of B cells secreting ACPA by microengraving

Microengraving is a soft-lithographic process for printing protein arrays, where each spot on the array comprises the proteins secreted by a single cell [35,36]. This technology was first used to isolate hybridomas producing hmAb and also to identify antigen-specific primary B cells from humans [11,35]. Here, we describe microengraving for screening, identification and isolation of ACPA secreted from B cells in a rapid and high-throughput manner. The nanowell array is molded into a thin slab of polydimethylsiloxane (PDMS) and is able to isolate large numbers of B cells (~100,000). The array is then placed in contact with a glass slide coated with antigen to locally capture auto-antibodies during a 2 h period. Subsequently, the secreted auto-antibodies printed onto the glass slide are revealed using a secondary anti-human IgG/IgM/IgA conjugated to a fluorophore (Alexa Fluor, Molecular Probes, Grand Island, NY) and then imaged on a microarray scanner. The result is a footprint of isolated spots, each corresponding to the individual nanowell containing a single B cell secreting ACPA (Fig. 2B). Soluble mouse IgG is used to facilitate the registration of the nanowell array on the printed glass slide. In parallel, the nanowell array is imaged by fluorescence microscopy to enable detection of the B cell surface marker CD19, and the live-cell marker, Calcein Violet (Invitrogen).

After compiling and analyzing the combined data from both the microscopy images and the printed glass slide, quantitative single-cell analysis tables are constructed to determine the location, frequency and phenotype of the B cells secreting ACPA (Fig. 2B). This information is then used to guide the retrieval of single cells.

In a routine experiment, the frequency of B cells secreting ACPA detected is 0.02–0.10% of single B cells.

2.4.1. Preparation of nanowell arrays

A "master" template, patterned using photolithography, is used as a mold to print elastomeric PDMS arrays that conform to the dimensions of a standard microscopy slide (25 × 75 mm). Detailed protocols for the design and fabrication of the master are available elsewhere (Fig. S1)[37,38]. Before use, the PDMS nanowell array is sterilized and rendered hydrophilic by brief exposure to air plasma.

1. Mix thoroughly the Sylgard 184 elastomer kit base and curing agent (Dow Corning, Midland, MI) at 10:1 weight ratio in a disposable cup using a plastic knife.
2. Degas the mixture in a vacuum chamber for 1 h.
3. Pour mixture onto the master, seal with a glass slide and let it sit for 30 min.
4. Transfer the assembly into an oven set to 80 °C for 2 h to cure the PDMS and bond it to the glass slide. Then let it cool at RT for 1 h.
5. Carefully lift off the glass slide from the silicon master containing the PDMS nanowell array, and cover the chip with Scotch tape until use.

2.4.2. Coating of auto-antigen on poly-L-lysine glass slides

1. Prepare capture antibody solution by adding 25 μg/mL of Streptavidin (Sigma) and 10 μg/mL of goat anti-mouse Ig (Southern Biotech, Birmingham, AL) in 80 μL of borate buffer (50 mM sodium borate, 50 mM sucrose/trehalose, 80 mM NaCl, pH 9).
2. Pipet the solution on the poly-L-lysine-coated glass slide and gently place a cover slip to spread uniformly on the slide. Incubate for 1 h at RT in a humidified chamber.
3. Block with 10 mL of 10% BSA in PBST for 2 h at RT.
4. Wash the slide with PBST for 5 min and then with PBS for 5 min. Rinse quickly with deionized (DI) water and dry the slide in a microarray centrifuge.
5. Add with 80 μL of 5 μg/mL biotinylated Cyclic-Citrullinated Peptide (Anaspec) and gently place a cover slip to spread uniformly on the slide.
6. Incubate for 1 h at RT.
7. Block with 10 mL of 3% BSA PBST for 10 min at RT.
8. Repeat step 4 and store in a humidified Petri dish until further use.

Note: The glass slide can be stored overnight at 4 °C in a humidified chamber.

2.4.3. B cell loading

1. Oxidize the PDMS nanowell array using standard plasma cleaner for 1 min at high radiofrequency setting and place the array face-down in sterile PBS.
2. Count the B cells on a hemacytometer by trypan blue exclusion (Sigma). Re-suspend to 5×10^5 cells/mL in 300 μL of R10 media or approximately 0.5–1 × 10^6 cell/mL to avoid a large number of cells per well.
3. Flip the nanowell array face up and aspirate the PBS. Quickly re-immerse the array in 5 mL of R10 and let it stand for 5 min.

Note: Ensure that the chip does not dry out.

4. Aspirate the R10 and load the cells onto the nanowell array by dispensing them drop-wise and letting them settle for 5 min. Check the loading using a standard tissue-culture, inverted microscope and load again if necessary to achieve desired density (~1 cell/well average).
5. Remove excess cells by rinsing the array with 5 ml of RPMI-PLGH..

2.4.4. Microengraving

1. Rinse the nanowell array with RPMI-PLGH containing 5 ng/mL soluble mouse IgG.
2. Remove the excess media from the PDMS nanowell array along the edges until the outline of the microchannels appears.

Note: Adequate care needs to be taken to ensure that the cells are not aspirated directly from the nanowells.

3. Place the PDMS into the hybridization chamber (Agilent Technologies, Santa Clara, CA), and quickly place the coated glass slide face down over the nanowell array. Press gently and close the chamber tightly.
4. Incubate the assembled chamber in an incubator for 2 h at 37 °C/5% CO_2 to enable microengraving of the secreted proteins.
5. Transfer the sandwich (nanowell array + glass slide) in a Petri dish containing pre-warmed RPMI-PLGH media.
6. Carefully detach the glass slide from nanowell array and wash in 1% milk in PBST for 10 min at RT (for blocking).
7. Wash with PBST for 5 min and then PBS for 5 min. Rinse the glass slide with DI water and dry by centrifugation.
8. Prepare 80 μL of detection solution containing 1 μg/mL of anti-human IgG (Jackson Immunoresearch)-Alexa Fluor (AF) 488, anti-human IgA-AF 532 (BD, Franklin Lakes, NJ), anti-human IgM-AF 594 (BD),goat anti-mouse IgG 647 (Invitrogen) in PBS. Deposit on the glass slide and place a cover slip to uniformly coat the slide.
9. Incubate for 1 h at RT in humidified chamber and protected from dark.
10. Repeat step 7.
11. Image the slide on a microarray scanner like the GenePix 4200AL (Molecular Devices, Sunnyvale, CA). Setup the appropriate excitation wavelengths and emission filters to match the fluorescent dyes used in the experiment.

2.4.5. B cell labeling and imaging

1. Prepare 300 μL of 4 μg/mL anti-CD19-AF 532 (BioLegend, San Diego, CA) and 1 μg/mL Calcein violet-AM in PBS. Deposit on the surface of the array containing the B cells. Incubate with this staining solution for at least 30 min at 37 °C/5% CO_2 in the dark before imaging.

Note: The labeling can also be performed at 4 °C. Additional antibodies labeled with orthogonal dyes directed against other phenotypic markers like CD27, CD38 and CD20 can be included in this step.

1. Acquire images of the nanowell array using a fluorescence microscope such as Axio Observer Z-1 inverted microscope equipped with a motorized stage and Lambda-DG4 illumination system (Zeiss, Jena, Germany).
2. After imaging, place the nanowell array into a 4-well plate and immobilize it by adding 2% agarose on its glass edges (top and bottom). Immerse in cold PBS and carefully float the coverslip off the array.
3. Store the nanowell array containing B cells at 4 °C until single cell retrieval by micromanipulation.

2.4.6. Data analysis

Data tables that report the phenotype (anti-CD19-AF 532 and calcein violet) and the location of every single B cell on-chip are obtained using image segmentation routines to automatically process the microscopy images.

Separately, the microengraved images are analyzed using appropriate software packages (Genepix Pro 6.1, Molecular Devices) to tabulate the fluorescent intensities of each Ig-positive spot within the array. Standard database matching algorithms are then employed to correlate the two sets of tables to identify the locations of nanowells containing live B cells secreting ACPA.

Once the analysis is complete, the information is exported to the manipulator for B-cell retrieval (Fig. S2).

2.4.7. Automated retrieval of antigen-specific B cells

1. Prepare collection tubes by adding 2 μL of RT buffer 5× (Invitrogen) and 2 μL of sterile nuclease-free water in 0.1 mL PCR tubes (Axygen, Union City, CA).
2. Place the 4-well plate containing the nanowell array on the motorized stage in the CellCelector microscope (ALS, Jena, Germany).
3. Prepare the CellCelector micromanipulator by loading the .CSV file containing the locations of ACPA secreting B cells (picking positions) and by calibrating the *X*, *Y* and *Z* positions.
4. Start the picking and ensure that the desired cells have been retrieved by checking the brightfield images.
5. Stored the retrieved B cells at −80 °C until further use.

Note: The frozen cells can be stored at −80 °C indefinitely.

2.5. Single cell RT-PCR and amplification

After single B cell isolation, RT-PCR is performed based on previously published protocols with minor modifications [39] (Table 1). Lysis of single cells to yield the mRNA is facilitated by freeze–thawing and the addition of detergents. The cDNA is synthesized using a primer mixture complementary to V_H or V_L chain constant region sequences. Rounds of first and second PCR amplification are performed by using the appropriate primer mixes (Integrated DNA Technologies, Coralville, IA).

We have successfully amplified immunoglobulin heavy and light chains from auto-reactive single B cells. Typical success rates are ~30–50% for amplification of pairs of variable heavy and light chains (V_H:V_L).

1. Thaw the PCR tubes containing single memory B cells and store them on ice.
2. Add 3 μL of 5% NP-40 (Sigma) and 1 μL of 5 pmol/μL of constant region RT primer mix (Table 1). Place the PCR tubes in a thermocycler (Applied Biosystems, Carlsbad, CA) with heating at 65 °C for 3 min, followed by cooling at 25 °C for 3 min. Store the tubes on ice.
3. Add 2 μL of RT buffer 5×, 2 μL of DTT (Invitrogen), 1 μL of 10 mM dNTP (Takara, Shiga, Japan) and 0.5 μL of 200 U/μL Superscript III RT (Invitrogen) to each tube, up to a final volume 19.5 μL.
4. Place the tubes into the thermocycler (Applied Biosystems) and heat at 37 °C for 1 h for first strain cDNA synthesis and then heat to 70 °C for 10 min to inactivate the enzyme.
5. Use the cDNA synthesized in the previous step as template for the first PCR. Take two aliquots of 8 μL cDNA products to amplify V_H and V_L. To one sample aliquot of 8 μL cDNA product,

Table 1
Primer sequences used for reverse-transcription and subsequent amplification of the variable regions from single B cells.

Description	Name	Sequence
cDNA synthesis		
Constant region RT primer mix	CμI	GCAGGAGACGAGGGGGA
	CγI	AGGG(C/T)GCCAGGGGGAA
	CκI	AACAGAGGCAGTTCCAGA
	Cλ	AC(C/T)AGTGTGGCCTTGTTGG
	Cα	GAGGCTCAGCGGGAAGAC
First PCR		
VH leader sequences		
OH II	VHL-1	TCACCATGGACTG(C/G)ACCTGGA
	VHL-2	CCATGGACACACTTTG(C/T)TCCAC
	VHL-3	CCATG GAR TT(C/T)GGG CTG AGC TGG
	VHL-4	AGAACATGAAACA(C/T)CTGTGGTTCTT
	VHL-5	ATGGGGTCAACCGCCATCCT
	VHL-6	ACAATGTCTGTCTCCTTCCTCAT
OLλ II*	VλL-1	GGTCCTGGGCCCAGTCTGTGCTG
	VλL-2	GGTCCTGGGCCCAGTCTGCC
	VλL-3	ATGGCCTGGA(C/T)C(C/G)CTCTCC
	VλL-4/5	GGTCTCTCTCSCAGC(C/T) TGTGCTG
	VλL-6	GTTCTTGGGCCAATTTTATGCTG
	VλL-7	GGTCCAATTC(C/T)CAGGCTGTGGTG
	VλL-8	GAGTGGATTCTCAGACTGTGGTG
OLκ II	VκL-1/2	ATGAGG(C/G)TCCC(C/T)GCTCAGCTGCTGG
	VκL-2	CTGGGGCTGCTAATGCTCTGG
	VκL-3	TTCCTCCTGCTACTCTGGCTC
	VκL-4	CAGACCCAGGTCTTCATTTCT
Constant regions sequences	CμII	CAGGAGACGAGGGGGAAAAG
	CλII	AGCTCCTCAGAGGAGGG(C/T)GG
	CκII	TTTCAACTGCTCATCAGATGGCGG
	CγII	GCCAGGGGGAAGAC(C/T)GATG
	CαII	GCTCAGCGGGAAGACCTT
Nested PCR		
OH III	VH-1	TTGCGGCCGCCAGGT(G/C)CAGCTGGT(G/A)CAGTC
	VH-2	TTGCGGCCGCCAG(A/G)TCACCTTGAAGGAGTC
	VH-3	TTGCGGCCGC(G/C)AGGTGCAGCTGGTGGAGTC
	VH-4	TTGCGGCCGCCAGGTGCAGCTGCAGGAGTC
	VH-5	TTGCGGCCGCGA(G/A)GTGCAGCTGGTGCAGTC
	VH-6	TTGCGGCCGCCAGGTACAGCTGCAGCAGTC
OLκ III	Vκ-1	CATAAGATCTCG(A/C)CATCC(A/G)G(A/T)TGACCCAGT
	Vκ-2	CACCAGATCTCGAT(A/G)TTGTGATGAC(C/T)CAG
	Vκ-3	ACCAGATCTCGAAAT(T/A)GTG(T/A)TGAC(G/A)CAGTCT
	Vκ-4	CACCAGATCTCGACATCGTGATGACCCAGT
OLλ III	Vλ-1	TATTAGATCTCCAGTCTGTGCTGACTCAGC
	Vλ-2	TATTAGATCTCCAGTCTGCCCTGACTCAGC
	Vλ-3	CACCAGATCTCTCCTATGAGCTGAC(T/A)CAGC
Nested constant regions	CμIII	AGGTCTAGAGAAAAGGGTTGGGGCGGATGC
	CγIII	AGGTCTAGAGAC(C/G)GATGGGCCCTTGGTGGA
	CκIII	TATTCCATGGAAGATGAAGACAGATGGTGC
	CλIII	CATTCCATGGGGGAACAGAGTGACCG
	CαIII	GACCTTGGGGCTGGTCGGGGA

add 6 μL of PCR buffer 10×, 1.6 μL of 10 mM dNTP (Takara, Shiga, Japan), 0.5 μL of leader sequence primer mixture (containing 20 pmol/μL of each primer), 0.5 μL of constant region primer mixture (20 pmol/μL of each primer), 1 μL of 2.5 U/μL Taq Polymerase (Takara, Shiga, Japan) and 48 μL of H_2O.

Note: In order to conserve reagents, we apply a tiered approach to the amplification of single cells. The V_H regions are amplified first and V_L region amplification is only performed for those samples from which we could identify successful V_H amplicons on an agarose gel (Fig. 3).

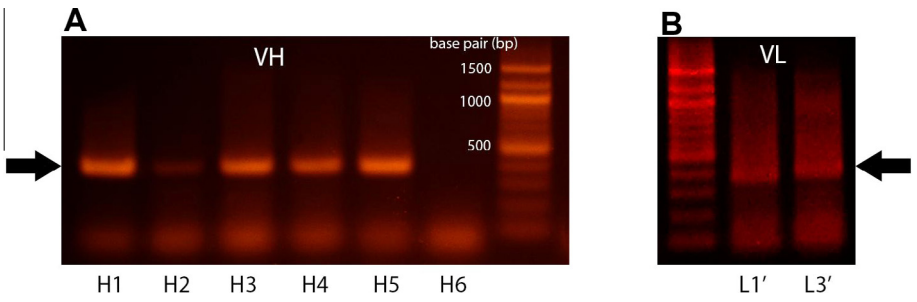

Fig. 3. Amplification of the V_H and V_L regions from single B cells. Subsequent to cell lysis and reverse transcription to yield cDNA, two rounds of PCR are employed to amplify the V_H and V_L regions. The PCR products are run on an agarose gel and visualized by ethidium bromide staining (~400 bp, dark arrow). (A) Lanes H1–H6 and (B) L1′–L3′ show gene amplification for V_H and V_L products, respectively, from 6 single B cells. Sequential amplification of V_H and then the V_L is performed to conserve reagents.

1. Program 3 cycles of pre-amplification at 94 °C for 45 s, 45 °C for 45 s and 72 °C for 1 min 45 s. Next, perform 30 cycles of amplification at 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1 min 45 s and complete by incubation at 72 °C for 10 min.
2. Prepare the nested PCR mix by transferring 3 μL of first PCR product to a new tube and adding 5 μL of PCR buffer 10×, 1.25 μL of 10 mM dNTP, 1 μL of nested PCR primer mixture (20 pmol/μL of each primer), 1 μL of constant region III primer (20 pmol/μL of each primer), 1 μL of 2.5 U/μL Taq Polymerase and 38 μL of H_2O.
3. Run the PCR reaction using the following conditions: 30 cycles of amplification at 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1 min 45 s and final incubation at 72 °C for 10 min.
4. The DNA products are separated using agarose gel electrophoresis. Briefly, 3 μL of PCR product is mixed with 7.5 μL of Tris–acetate–EDTA (TAE) 1× buffer and loaded into 0.8% (wt/vol) agarose gel (Gibco, Grand Island, NY) with 10 μL of 1 kb DNA ladder (NEB, Ipswich, MA) and run at 110 V for 40 min. After staining for 20 min with 0.5 μg/mL ethidium bromide dye (NEB, Ipswich, MA) in TAE buffer, the gel is imaged by using UV light assisted visualization of bands at ~400 bp.
5. The PCR product is then used as template for further re-amplification to facilitate DNA sequencing and recombinant cloning, essentially as described previously [14]. Transient transfection into human kidney epithelial cells (HEK293) is used for expression of full-length hmAb and ELISA of the supernatants/purified proteins performed to confirm the antigen-specificity and of these antibodies. Subsequent to amplification, around 30–40% of antigen-specific antibodies selected using microengraving did not clone or express as full-length human IgG1 after transfection (unpublished data).

3. Conclusions

Here, we describe a technology to screen and isolate auto-antibodies derived from single B cells originating from the PBMC of RA patients. The entire procedure including the stimulation, microengraving, retrieval and amplification of cells can be accomplished in 1 week. The advantages of using microengraving are the ability to detect low numbers of candidate B cells secreting specific antibodies needed to isolate the auto-reactive hmAbs and the ability to determine the phenotype of these auto-reactive B cells.

We anticipate that this technology can be readily adapted for the screening and isolation of other auto-reactive antibodies to help elucidate their molecular contribution in pathology of autoimmune diseases and also determine their phenotype to facilitate therapeutic intervention.

Acknowledgments

This publication was supported by the National Cancer Institute of the National Institutes of Health under Award Number R01CA174385 and the University of Houston New Faculty Award (I102560). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. We thank Vandana Kaul and Balakrishnan Ramesh for critical comments on the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ymeth.2013.06.018.

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