A Novel Cell-Based Multiplex Immunoassay Platform

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A Novel Cell-Based Multiplex Immunoassay Platform

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Abstract

We describe generation of stable, fluorescence-barcoded cell lines suitable for multiplex screening of antibody to membrane proteins. The utility of this cell-based system, capable of a 256-plex cell panel, is demonstrated by flow cytometry deconvolution of barcoded cell panels expressing influenza A hemagglutinin trimers, or native human CCR2 or CCR5 multi-spanning proteins and their epitope-defining mutants. This platform may prove useful for characterizing immunity and discovering antibodies to membrane-associated proteins.

Main

Membrane proteins are often critical immunogens in humoral responses to infection and physiologically significant in health and disease. They are, therefore, valuable targets in vaccine design and therapeutic antibody development[1]. For many multi-subunit or multi-pass transmembrane proteins, the native conformation is preserved only when the protein is properly expressed on the cell membrane[2]. For this reason, cell-based binding assays using protein antigens expressed on the cell-surface are widely used in antibody screening and characterization[3]. A major limitation in these assays is that they are single-analyte, or singleplex methods, and consequently time-consuming, sample-depleting and costly when used to screen samples with high numbers (>10) of antigens. While multiplex immunoassays based on the Luminex® technology are well established, only acellular antigens can be immobilized on detection beads with this platform.

Here, we describe a cell-based, multiplex immunoassay to fill this technology gap. Briefly, a basal cell line was engineered to express different combinations of fluorescent proteins (FPs) that can be reliably detected and distinguished in different channels on flow cytometers. This resulted in a panel of fluorescence-barcoded reporter cell lines analogous to barcoded Luminex® beads. Individual reporter cell sub-lines, each bearing a unique combination of FPs, were subsequently engineered to express different cell-surface proteins to permit their pooling in a cell-based multiplex immunoassay. Flow cytometry was then used to de-multiplex signals from pooled reporter cell lines by serial gating for the identifying FP combinations. In this proof-of-concept study, we established a 16-plex basic panel and tested the feasibility of expanding to a 256-plex panel of reporter cell lines. We successfully applied this novel platform to multiplex detection of antibody binding to cell panels expressing various influenza A hemagglutinin (HA) trimers, or human CCR2b and CCR5 and several domain-swap or point mutants that define specific protein domains/epitopes.

The human chronic myelogenous leukemia cell line K562 was selected as a parental cell line; as a suspension cell line, detachment or digestion treatment is unnecessary, removing the possibility of denaturation of expressed surface antigens. K562 cells also exhibit a high efficiency of transfection and transduction, and are resilient to apoptosis[4], simplifying the processes of genetic modification and assay processing. A drawback of K562 as a reporter cell line is its expression of the Fcγ receptor, CD32A, which binds most human IgG subclasses with sufficient affinity to raise background thresholds (Supplementary Fig. 1). We therefore knocked-out *CD32A* by CRISPR-Cas9 mediated gene targeting (Supplementary Fig. 1). Using genomic

DNA sequencing to ensure disabling all *CD32A* alleles, we generated a cloned, basal cell line, K530. Compared with the parental K562 cells, K530 cells are negative for all Fcγ receptors CD16, CD32, and CD64 and show minimal non-specific binding by human IgG1 (Supplementary Fig. 1).

To introduce unique barcodes into the reporter cells for multiplex detection, we conceived a strategy to use combinations of FPs expressed in the cytosol of reporter cells. The growing toolbox of FPs[5] coupled with widely used multicolor flow cytometry allows for marking and detecting multiple FPs simultaneously. The number of unique FP combinations is 2^n where n is the number of different FPs. This exponential result provides the "power" for multiplexity. After careful selection and experimental tests, we selected eight FPs (EBFP2[6], mTurquoise2[7], mNeonGreen[8], mCardinal[9], mKate2[10], miRFP703[11], LSSmOrange[12] and hmKeima8.5[13]; Supplementary Table 1) with features including: *1*) bright fluorescence for good separation and limited spill-over into other fluorescence channels, *2*) good photostability and low cytotoxicity, and *3*) monomeric FPs to avoid potential FRET events between heterologous FPs. We designed and generated a four-color basic panel (Supplementary Fig. 2,3) capable of 16 distinct FP combinations. Extended panels with two (Supplementary Fig. 2) or four additional colors (Supplementary Fig. 3) could expand the multiplexity to 64- or 256-plex, respectively. Alternatively, increased multiplexity could be achieved by introduction of a reference membrane protein (*e.g.*, CD8a) or by high/low intensity versions of the same FPs (Supplementary Fig. 4).

We validated the four-color basic panel in supporting 16-plex detection of cell-surface molecules. K530 cells were engineered to express all 16 combinations of four FPs from the basic panel, resulting in 16 uniquely fluorescence-barcoded cell lines (Fig. 1a). Pooled cell lines can be demultiplexed by flow cytometry based on patterns of FP expression (Fig. 1b). To validate the deconvolution of multiplexed cell populations, we generated 16 cell lines expressing human CD4, CD8a, CD86, or CD154 so that each protein was associated with four unique FP patterns. The pooled cells were stained in single tubes with monoclonal antibody for one of the four human antigens, followed by a common PE-conjugated secondary antibody. Deconvolution by flow cytometry showed high resolution of bound and unbound cells and patterns of binding consistent with antigen expression by barcoded cells before multiplexing (Fig. 1c, Supplementary Fig. 5).

Influenza hemagglutinins (HAs) are diverse trimeric membrane proteins. Recombinant HA protein preparation can be challenging as some HA ectodomains are unstable, and cell-surface expression can be a surer option for obtaining native structure when working with unknown or novel influenza strains. Multiplex immunoassays with collections of cell-surface expressed HA antigens may be useful in influenza vaccine design and immunological monitoring. To demonstrate, we prepared a panel of 12 reporter cell lines expressing the HA trimers of diverse influenza A subtypes and successfully validated their structural integrity with several reference antibodies (Supplementary Fig. 6). The pan-influenza A stem-binding antibody FI6[14] bound to all the HA antigens tested. The S5V2-29 antibody specific for a conserved interface epitope[15] bound to a majority of group 1 and group 2 HAs tested and CH67, a neutralizing antibody for H1N1 influenza[16] bound HA from A/Solomon Islands/3/2006(H1N1) but not

A/Christchurch/16/2010(H1N1), an A/California/07/2009(H1N1)-like strain. Antibody HC19, specific for HA A/Hong Kong/JY2/1968(H3N2)[17] bound to that HA but not to others. We have also successfully applied this assay in high-throughput screening of immunogen-specific antibodies with single memory B cell cultures from influenza-vaccinated subjects (unpublished).

G protein-coupled receptors (GPCRs) are key to many biological functions and are an important class of drug targets, contributing 34% of FDA approvals[18]. For these seven-pass transmembrane proteins, mammalian cell surface expression is the most reliable way to preserve native conformation. Domain/epitope mapping of GPCR-specific antibodies can be helpful for structure-function studies of the receptors and for the screening antibodies for therapeutic, diagnostic, and research applications[19]. A cell-based multiplex immunoassay that could accommodate the large number of protein variants needed for domain/epitope mapping in a single-tube could be useful. As an example, a panel of reporter cell lines was prepared, expressing human CCR2b and CCR5 and 12 domain-swapped chimeric mutants (Fig. 2a). Two CCR2-specific and five CCR5-specific antibodies were tested for their binding patterns to these domain-swapped mutants in a multiplex immunoassay (Fig. 2b). We found the two uncharacterized CCR2 antibodies to be specific for the CCR2 N1 domain (Fig. 2b,c). Of the previously characterized CCR5 antibodies, CTC8, 2D7 and 45529 bound to the N1, ECL2A and ECL2B domains, respectively, whereas binding of 45523 and 45549 was not isolated to any single domain (Fig. 2b,c). These observations matched prior reports[20, 21]. The combination of gain- and loss-of-binding readouts across all the ectodomains allowed a semi-quantitative determination of the contribution of each individual ectodomain (Fig. 2c) and revealed more minor-contributing domains unappreciated previously[20, 21]. Epitope mapping with another panel of reporter cell lines expressing CCR5 mutants with alanine scanning (or glycine-to-serine mutation) in ECL2A domain (Fig. 2d,e) showed that, consistent with previous studies[20, 21], K171 and E172 were critically involved in the binding by 2D7 and 45523. With this scanning panel, another five residues were also determined to be engaged in antibody binding, which were not covered or appreciated in previous studies. The only discrepancy with previous studies[20] is the role of K171 and E172 in the binding by 45549 and awaits further confirmation. In practice, a full scanning panel can be established to cover every residue in all ectodomains. This multiplex immunoassay can enable high-throughput screening of antibodies with desired specificity and potential function at single-residue resolution in a single-tube manner and expedite therapeutic or diagnostic antibody discovery.

In summary, we have developed a cell-based multiplex immunoassay platform complementary to the well-established Luminex® platform with acellular antigens. We validated its feasibility in multiplexing and reliability in demultiplexing by flow cytometry. We demonstrated its broad application in multiplex detection of antibody binding to influenza HA and domain/epitope mapping against human CCR2b/CCR5 mutant antigens. The cell-based property of this platform offers flexibility in multiplexing, superiority in supporting surface display of complex membrane proteins in their native conformation, and economy in production once corresponding cell lines are established. These unique features make this novel platform a valuable tool in humoral response monitoring and antibody screening and characterization.

Methods

Mice and human subjects

Female C57BL/6 mice were obtained from the Jackson Laboratory and maintained under specific-pathogen-free conditions at the Duke University Animal Care Facility. Splenocytes were isolated from one 12-week-old mice for RNA extraction. All experiments involving animals were approved by the Duke University Institutional Animal Care and Use Committee (IACUC A128-20-06).

Peripheral blood mononuclear cells (PBMCs) were obtained from one human (*Homo sapiens sapiens*) subject under Duke Institutional Review Board Committee guidelines (IRB Pro00062495). Written informed consent was obtained.

Original plasmids and DNA templates for gene cloning

pU6-(BbsI)_CBh-Cas9-T2A-BFP (Addgene plasmid # 64323) was a gift from Ralf Kuehn[22]. pLV-EF1a-IRES-Puro (Addgene plasmid # 85132) was a gift from Tobias Meyer[23]. pLB (Addgene plasmid # 11619) was a gift from Stephan Kissler[24]. pMD2.G (Addgene plasmid # 12259) and psPAX2 (Addgene plasmid # 12260) were gifts from Didier Trono. mScarlet-I-mTurquoise2 (Addgene plasmid # 98839) was a gift from Dorus Gadella[25]. pLenti-smURFP-T2A-mCardinal (Addgene plasmid # 80348) was a gift from Erik Rodriguez & Roger Tsien[26]. pMito-miRFP703 (Addgene plasmid # 80000) was a gift from Vladislav Verkhusha[11]. pLSSmOrange-mKate2 (Addgene plasmid # 99868) was a gift from Marc Tramier[27]. Plasmid mNeonGreen-C1 was provided by Allele Biotechnology and Pharmaceuticals Inc. under a license for non-commercial use.

DNA template coding fluorescent protein (FP) hmKeima8.5 was synthesized (Genscript) based on published amino acid sequences[13]. Coding sequences for other FPs were cloned from plasmids mentioned above, with EBFP2[6] from pU6-(BbsI)_CBh-Cas9-T2A-BFP, mTurquoise2[7] from mScarlet-I-mTurquoise2, mNeonGreen[8] from mNeonGreen-C1, mCardinal[9] from pLenti-smURFP-T2A-mCardinal, mKate2[10] and LSSmOrange[12] from pLSSmOrange-mKate2 and miRFP703[11] from pMito-miRFP703. Coding sequences for cytoplasmic domain truncated mouse CD4 (amino acid (aa) 1-423), CD8a (aa 1-222) and CD86 (aa 1-268) were cloned from splenocyte cDNA samples from one C57BL/6 mouse. Coding sequences for full length human CCR2b and CCR5, and cytoplasmic domain truncated human CD4 (aa 1-424), CD8a (aa 1-209), CD86 (aa 1-276) and CD154 (aa 1-240) were cloned from PBMC cDNA samples from one healthy donor.

DNA templates coding influenza hemagglutinin (HA) antigens A/Christchurch/16/2010(H1N1) (GISAID Accession EPI280344) and A/Texas/50/2012(H3N2) (GenBank Accession KJ942744) were synthesized (Gene Universal Inc.) based on amino acid sequences in the databases. Plasmid containing coding sequences for influenza HA A/Solomon Islands/3/2006(H1N1) (GenBank EU100724, with R540L correction) was provided by Aaron G. Schmidt (Ragon Institute). Plasmids containing coding sequences for other influenza HA antigens used were provided by Stephen C. Harrison (Harvard Medical School), including HA antigens A/Japan/305/1957(H2N2) (GenBank CY014976), A/Hong Kong/JY2/1968(H3N2) (GenBank CY147438), A/American black duck/New Brunswick/00464/2010(H4N6) (GenBank CY138045),

A/Viet Nam/1203/2004(H5N1) (GenBank AY818135), A/Taiwan/2/2013(H6N1) (GISAID EPI459855), A/Taiwan/1/2017(H7N9) (GISAID EPI917065), A/northern shoveler/California/HKWF1204/2007(H8N4) (GenBank CY039588), A/Jiangxi/IPB13/2013(H10N8) (GenBank KJ406543) and A/mallard/Wisconsin/10OS3941/2010(H14N6) (GenBank CY133266).

Plasmid modification and gene cloning

Standard molecular cloning procedures were followed for plasmid modification and gene cloning. Endotoxin-free plasmids were prepared (E.Z.N.A.® Endo-free Plasmid DNA Mini Kit II, Omega Bio-tek) for mammalian cell transfection. Recombinant sequences in all plasmids used were verified by DNA Sanger sequencing (The Duke University DNA Analysis Facility).

The empty lentiviral transfer vector plasmids pLB-EF1a, pLB-EFS and pLB-EF1a-IRES-Puro (pLB-EXIP; IRES stands for internal ribosome entry site) were constructed by replacing the U6-loxP-CMV-EGFP-loxP cassettes in plasmid pLB with EF1a promoter, EFS core promoter (nucleotide 1-226 of EF1a promoter, with attenuated transcription activity) or EF1a-IRES-Puro cassettes from plasmid pLV-EF1a-IRES-Puro, respectively. pLB-EXIP was further modified to generate bicistronic vectors pLB-EF1a-IRES-EBFP2, pLB-EF1a-IRES-mTurquoise2, pLB-EF1a-IRES-mCardinal, pLB-EF1a-IRES-mCD4, pLB-EF1a-IRES-mCD8a, pLB-EF1a-IRES-mCD86 by replacing puromycin-resistant gene in pLB-EXIP with coding sequences for corresponding genes following IRES element. pLB-EFS-IRES-mNeonGreen was generated from pLB-EXIP by replacing EF1a promoter with EFS core protomer and puromycin-resistant gene with mNeonGreen coding sequences.

Lentiviral transfer vector plasmids expressing EBFP2, mTurquoise2, mCardinal, mKate2, miRFP703, LSSmOrange, hmKeima8.5 were generated by cloning of corresponding coding sequences into pLB-EF1a. Lentiviral transfer vector plasmid expressing mNeonGreen was generated by cloning of mNeonGreen coding sequences into pLB-EFS. Human CD4, CD8a, CD86 and CD154 coding sequences (with cytoplasmic domain truncations, see above) were cloned into pLB-EXIP for puromycin-mediated selection. Human CD8a coding sequences were cloned into pLB-EF1a-IRES-mTurquoise2 and pLB-EF1a-IRES-mCardinal, and human CD86 coding sequences were cloned into pLB-EF1a-IRES-EBFP2 and pLB-EFS-IRES-mNeonGreen. These bicistronic expression vectors can co-express human CD8a/CD86 and FPs, with FPs at a lower expression level driven by IRES element. Coding sequences for influenza HA antigens, human CCR2b, CCR5 and their domain-swapped or point mutants were cloned into pLB-EXIP.

Culture, transfection and transduction of mammalian cell lines

HEK 293T and K562 cell lines were purchased from ATCC. HEK 293T cells were cultured in DMEM medium (Gibco) supplemented with 10% heat-inactivated HyClone FBS (Cytiva), 10 mM HEPES buffer and 55 μM 2-Mercaptoethanol (all Gibco). K562 and derivative cell lines were maintained in RPMI-1640 medium (Gibco) supplemented with 10% heat-inactivated HyClone FBS, 10 mM HEPES buffer, 1 mM sodium pyruvate, 1× MEM NEAA, 55 μM 2-Mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin (all Gibco). For all K562 derivative cell lines,

monoclonal cell lines were established by single-cell sorting (see below) and used in binding assays in this study.

Single-guide RNAs (sgRNAs) targeting human *CD32A* exon 1 were designed with the online tool (http://crispr.mit.edu). sgRNAs used in this study were sgRNA-hCD32A-1 (AGCAGCAGCAAAACTGTCAA), sgRNA-hCD32A-2 (ATGTATGTCCCAGAAACCTG) and a negative control sgRNA (TGTCATGCGTCACTTAGTGC). Corresponding DNA oligos were synthesized and cloned into plasmid pU6-(BbsI)_CBh-Cas9-T2A-BFP. K562 cells were transfected with CRISPR-Cas9 targeting plasmids using Lipofectamine 3000 Transfection Reagent (Invitrogen). 96 hours after transfection, the cells were harvested for flow cytometry analysis and single-cell sorting (see below).

Lentiviral transfer vector plasmids were co-transfected into HEK 293T cells with packaging plasmids pMD2.G and psPAX2 using Lipofectamine 3000 Transfection Reagent (Invitrogen). Forty-eight hours after transfection, culture supernatants were harvested and filtered through 0.45-µm PVDF membrane filters (Millipore). K562 derivative cell lines were transduced with the filtered supernatants containing lentiviral vectors by spinoculation at 1000× *g* for 45 min at 32°C. For transductions with pLB-EXIP-based vectors, cells were selected with puromycin (Sigma, 2 µg/ml) between 3-7 days after transduction. Seven days after transduction, the cells were harvested for flow cytometry analysis and single-cell sorting (see below).

Antibodies

Monoclonal antibodies used in this study included: FITC-conjugated anti-human CD32A (hCD32A-FITC, clone IV.3, STEMCELL 60012FI), hCD32-APC (clone FLI8.26, BD 559769), hCD16-PE (clone 3G8, BioLegend 302007), hCD32-PE (clone FLI8.26, BD 550586), hCD64-PE (clone 10.1, BioLegend 305007), hCD4 (clone SK3, BioLegend 344602), hCD8a (clone HIT8a, BioLegend 300902), hCD86 (clone BU63, BioLegend 374202), hCD154 (clone 24-31, BioLegend 310802), mCD8a-PerCP-eFluor710 (mouse CD8a, clone 53-6.7, ThermoFisher 46-0081), mCD86-PE-Vio770 (clone PO3.3, Miltenyi Biotec 130-105-135), mCD4-APC-Fire750 (clone RM4-5, BioLegend 100568), hCD58-PE (clone MEM-63, ThermoFisher MA1-10256), HA tag (clone 16B12, BioLegend 901533), hCCR2 (clone K036C2, BioLegend 357201), hCCR2 (clone 48607, R&D Systems MAB150), hCCR5 (clone 2D7, BD 555991), hCCR5 (clone CTC8, R&D Systems MAB1801), hCCR5 (clone 45523, R&D Systems MAB181), hCCR5 (clone 45529, R&D Systems MAB184), hCCR5 (clone 45549, R&D Systems MAB183). Isotype control antibodies included: Mouse IgG1 Kappa isotype control (Rockland 010-001-330), Human IgG1 Kappa (hIgG1K, Southern Biotech 0151K-01), Human IgG1 Lambda (hIgG1L, Southern Biotech 0151L-01). Secondary antibodies included: Goat Anti-Human IgG-PE (Southern Biotech 2040-09), Goat Anti-Mouse IgG, Human ads-PE (Southern Biotech 1030-09). Influenza hemagglutinin specific antibodies FI6[14], S5V2-29[15], CH67[16] and HC19[17] were prepared as recombinant human IgG1 antibodies as described[28].

Cell surface staining, flow cytometry analysis and single-cell sorting and cloning

Cultures of K562 and derivative cells were harvested, centrifuged at 300× *g* for 2 min at 4°C and resuspended in staining buffer (PBS supplemented with 2% heat-inactivated FBS). After

incubation with antibodies at 4°C in the dark for 30 min, cells were washed with staining buffer and resuspended in staining buffer for either secondary staining following the same procedure above or stored on ice for flow cytometry analysis or single-cell sorting.

Flow cytometry analysis was carried out using either BD FACSCanto II cytometer (Duke Cancer Institute Flow Cytometry Shared Resource) or BD LSR II cytometer (The Duke Human Vaccine Institute (DHVI) Research Flow Cytometry Facility). Single-cell sorting was performed with BD Aria II (The DHVI Research Flow Cytometry Facility). The bulk cell line after transfection or transduction were stained with corresponding antibodies and single cells expressing FP or antigen of interest were sorted into 96-well flat-bottom plates containing 100 µl/well of the complete RPMI medium above supplemented with 20% heat-inactivated FBS. Nine days after sorting, healthily proliferating cell clones were transferred into 24-well plates for further expansion. Three days later, individual monoclonal cell lines were validated for the expression of FP or antigen of interest and a single clone with a uniform expression level was selected for further engineering or used as a reporter cell line in immunoassays.

Data analysis

FlowJO (Version 10.7.2), Chromas (Version 2.6.6) and Microsoft Office (Version 1808) were used to analyze data and prepare figures for publication.

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Author Contributions

S.S. and G.K. conceived the project. S.S. and M.M. performed experiments. All authors analyzed the data and prepared the manuscript.

Competing Interests statement

Our institution, Duke University, has a patent application related to this work. Names of inventors are Shengli Song and Garnett Kelsoe. Application number is 63/033,444. Status of application is patent pending. The patent application covered the design and application of the novel cell-based immunoassay platform reported here.

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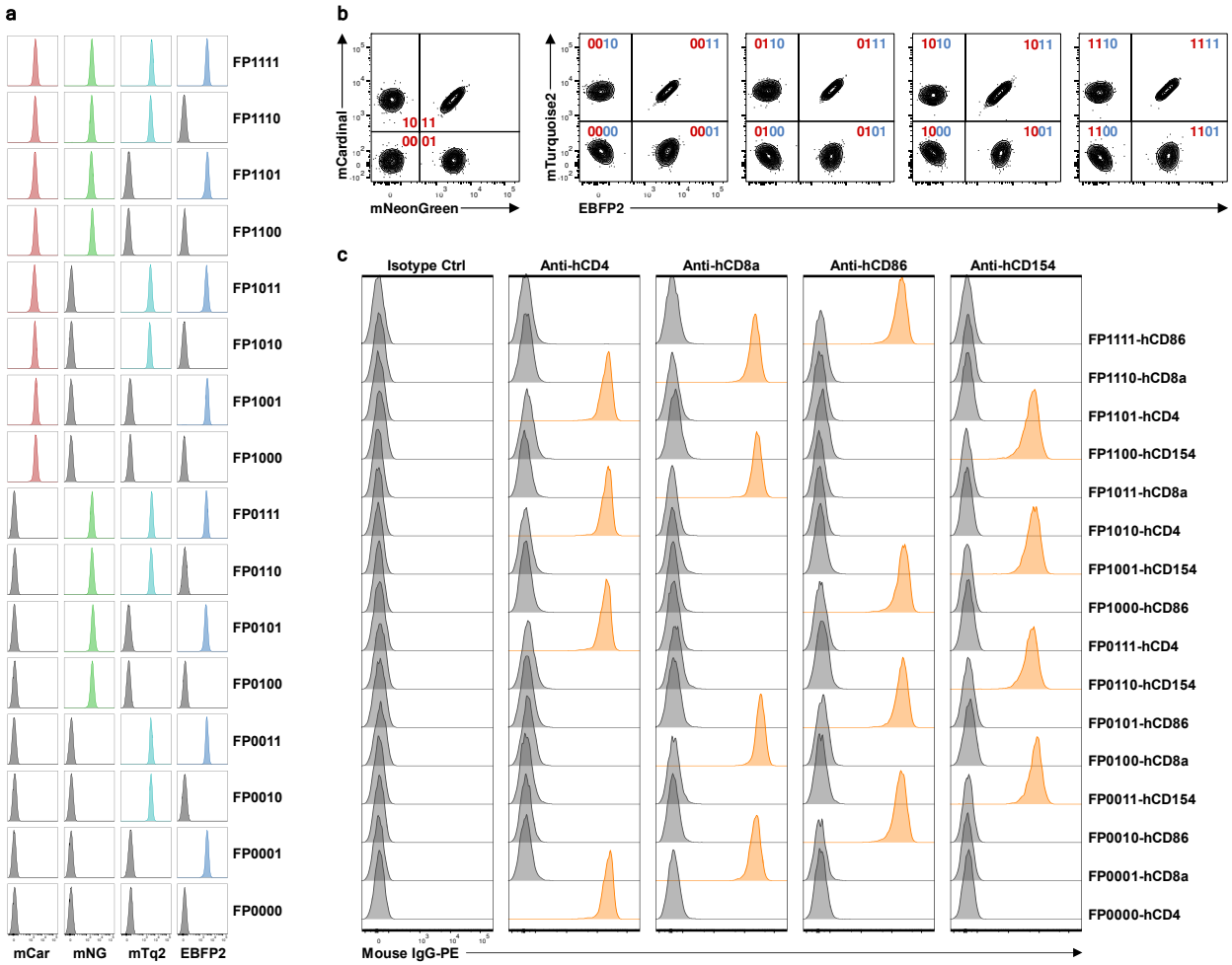

Figure 1. A multiplex immunoassay based on fluorescence-barcoded reporter cell lines. a, A basic panel of fluorescence-barcoded reporter cell lines. K530 cells were transduced with different combinations of four FPs to produce 16 uniquely fluorescence-barcoded cell lines. The absence/presence of fluorescence from FPs EBFP2, mTurquoise2 (mTq2), mNeonGreen (mNG) and mCardinal (mCar) are designated as four digits of binary barcodes as shown on the right of histograms for each individual cell line. **b**, Demultiplexing of pooled fluorescence-barcoded reporter cell lines by flow cytometry. **c**, The 16 barcoded reporter cell lines were transduced to express human CD4, CD8a, CD86 and CD154 molecules in a shifted pattern relative to FP expression. These cells were pooled and stained with corresponding mouse monoclonal antibodies (as indicated on the top of each histogram) followed by a PE-conjugated anti-mouse IgG antibody. Signals from individual reporter cell lines were demultiplexed as shown in **b**, and the binding by corresponding antibodies were plotted as half-offset histograms. In all cases, the detected expression patterns were consistent with antigen expression by barcoded cells before multiplexing as shown on the right of histograms for each individual cell line. Isotype Ctrl, mouse IgG1, kappa isotype control antibody.

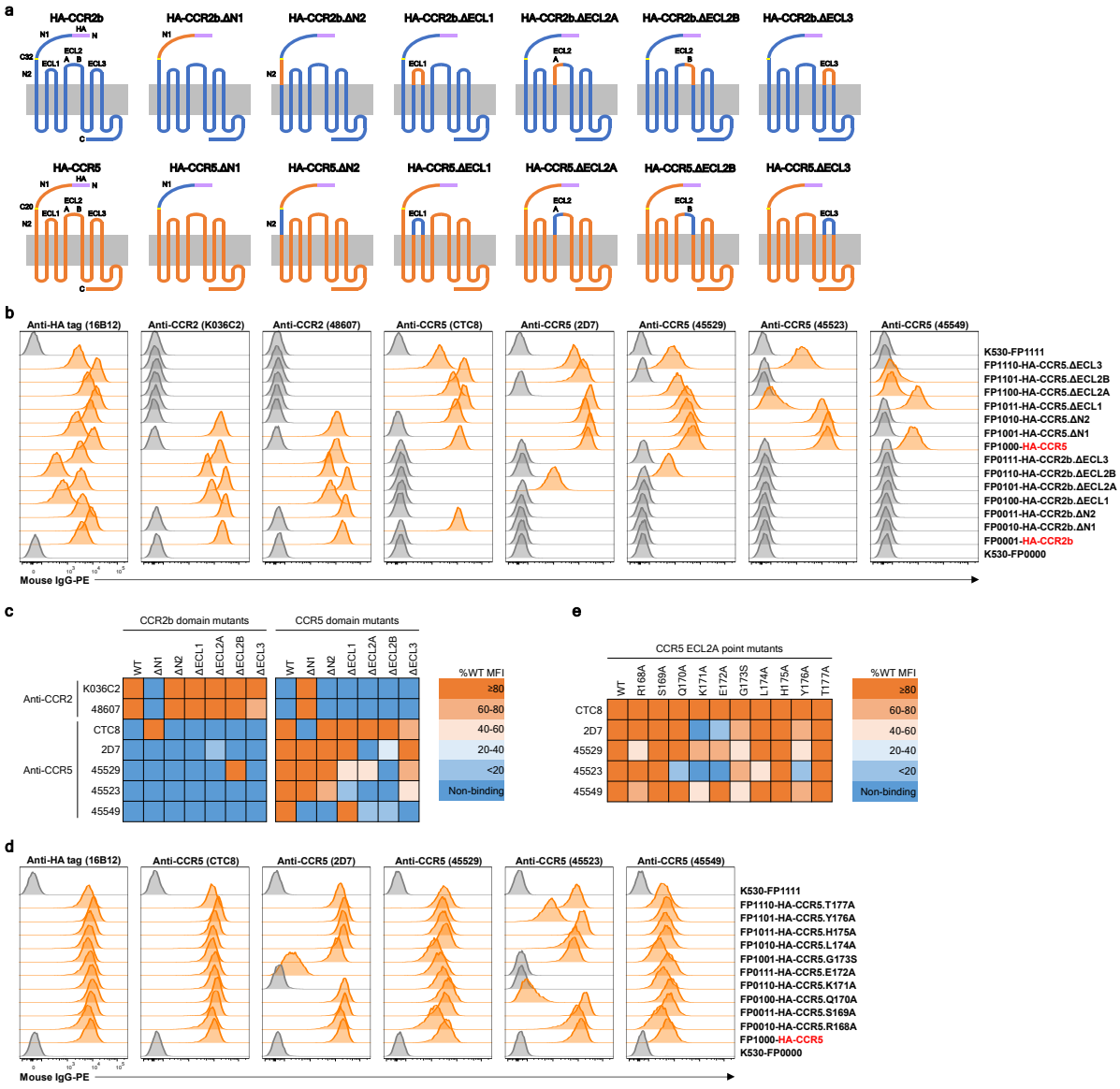

Figure 2. Application of the multiplex immunoassay in domain/epitope mapping of antibodies specific for human CCR2 and CCR5. a, Schematic diagrams showing CCR2b and CCR5 domain-swapped mutants expressed on reporter cell lines. An N-terminal HA-tag was included to normalize surface expression levels of different mutants. **b**, Domain mapping with multiplexed reporter cell lines expressing CCR2b and CCR5 domain-swapped mutants. The binding to each reporter cell line was demultiplexed as shown in Figure 1. Histograms with MFI values above 2-fold of background (the average MFI value of internal control cell lines K530-FP0000 and K530-FP1111) were scored as positive and highlighted in orange. **c**, Heat map of the relative binding activities of antibodies tested in **b**. Background-subtracted MFI values were first normalized to that with anti-HA-tag antibody and then to corresponding cell lines expressing WT molecules. Percentages of WT MFI values were color-coded according to the key. Negative readouts in **b** were indicated as non-binding. **d**, Epitope mapping with multiplexed reporter cell lines expressing CCR5 ECL2A point mutants. The data were analyzed in the same way as in **b**. **e**, Heat map of the relative binding activities of antibodies tested in **d** and plotted in the same way as in **c**. All monoclonal mouse antibodies were used at 5 μg/ml.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementary.pdf