

Measurement of Mast Cell Surface Molecules BY High-Throughput Immunophenotyping Using Transcription (HIT)

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Abstract

Here we describe the application of a highly multiplexed proteomic assay, called HIT (high-throughput immunophenotyping using transcription), to analyze human mast cell surface antigens at rest and during stimulation. HIT allows analysis of up to 100 analytes, including surface antigens and intracellular phosphoproteins, transcription factors, and cytokines, in a single experiment. Briefly, anti-mouse monovalent Fab fragments are covalently conjugated with barcoded oligonucleotides to generate a panel of conjugates. The oligonucleotide-Fab fragment conjugates are bound to monoclonal primary antibodies, creating a cocktail of up to 48 unique barcoded primary antibodies. As few as 100,000 mast cells are stained with the cocktail and the barcodes of the bound primary antibodies are amplified by *in vitro* transcription with fluorescently labeled NTPs. The resulting barcoded transcripts are quantified using a microarray spotted with oligonucleotides that are complementary to the barcoded transcripts. Differences in levels of the barcoded transcripts correlate well with actual protein levels and are capable of detecting stimulation-dependent changes in protein levels. HIT is an invaluable, broad-spectrum approach for characterizing mast cell surface antigens, signaling molecules, transcription factors, and cytokines.

Key words Mast cells, HMC-1, Microarray, Proteomics, Immunoassay, Multiplex, Surface marker profiling, Transcription, Monoclonal antibody

1 Introduction

Mast cells are innate immune effector cells, capable of releasing a wide variety of preformed and newly synthesized inflammatory and immunomodulatory molecules upon activation and degranulation. It is through the release of these mediators that mast cells provide protection against certain parasitic and bacterial infections, and also play a pathogenic role in allergy and anaphylaxis [1–3]. Mast cells are heterogeneous, expressing diverse phenotypes depending on their tissue of origin and microenvironment, and retain plasticity

between these phenotypes [4]. Further, the expression of mast cell mediators and surface molecules, response to stimuli, and level of activation are dependent on this heterogeneity [5]. Gene expression profiling of mast cells by microarray has been applied to evaluate these phenotypic differences [6]. This platform has the advantage of being highly multiplexed; however protein and transcript levels may differ significantly [6]. Traditional assays that measure protein levels directly have the disadvantage of only evaluating a few analytes. For example, analysis of mast cell cytokine production and signaling molecules by ELISA and western blot only measure a single analyte at a time. Conventional analysis of mast cell surface markers by flow cytometry measures a greater number of analytes in a single sample (~14 markers), but involves greater complexity in selection of compatible fluorescent dyes.

Our laboratory has developed a number of technologies that are directly applicable to proteomic analysis of mast cell biology, including reverse-phase protein lysate microarray, a technique that allows multiplexed analysis of hundreds of cell-signaling components in response to immune receptor signaling, and HIT (high-throughput immunophenotyping using transcription) [7–10]. HIT is a highly multiplexed, proteomic assay that allows analysis of up to 100 analytes, including surface antigens and intracellular phosphoproteins, transcription factors, and cytokines, in a single experiment. Here we have described the application of HIT to the analysis of human mast cell surface antigens at rest and during stimulation. Briefly, 5' amine-modified, barcoded oligonucleotides are modified to incorporate a formylbenzamide functional group at the 5' end of the oligonucleotides. Anti-mouse Fab fragments are concurrently treated with a linker to incorporate hydrazino-nicotinamide functional groups at lysine residues. The modified oligonucleotides and Fab fragments react to form antibody-oligonucleotide conjugates, covalently joined by a stable bis-arylhydrazone bond. Each unique antibody-oligonucleotide conjugate is bound to a monoclonal primary antibody, generating a cocktail of barcoded primary antibodies.

In our studies, a HIT cocktail of barcoded primary antibodies was used to stain stimulated HMC-1 cells, which were derived from a patient with mast cell leukemia [11]. The barcodes of the bound primary antibodies were amplified by *in vitro* transcription with fluorescently labeled NTPs. The resulting amplified barcoded transcripts were quantified using a microarray spotted with oligonucleotides complementary to each barcoded transcript. The markers identified by HIT were consistent with previously reported mast cell markers and were validated by flow cytometry. HIT represents an ideal approach for characterizing mast cell heterogeneity in surface antigens, signaling molecules, transcription factors, and cytokines.

2 Materials

Prepare all solutions using distilled-deionized water (ddH₂O) unless otherwise indicated.

2.1 Fab-Oligonucleotide Conjugation Components

1. Fab-oligonucleotide conjugation buffer: 25 mL of 100 mM sodium citrate (pH 5.75), 150 mM sodium chloride. Store at room temperature (RT).
2. Oligonucleotide annealing buffer: 5 mL of 10 mM sodium phosphate (pH 7.5), 100 mM sodium chloride. Store at RT.
3. Fab-oligonucleotide storage buffer: 25 mL of 50 % glycerol, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.05 % sodium azide (NaAz).
4. 30-mer oligonucleotide containing the T7 promoter sequence 5'-ATGGAATTCCTAATACGACTCACTATAGGG-3' with a 5' benzaldehyde modification (Trilink Biotechnologies, San Diego, CA).
5. 70-mer template strands containing barcode sequences flanked between T7 promoter and poly-adenylated tail sequences and 40-mer reverse complement sequences (Table 1) (Trilink Biotechnologies, San Diego, CA).
6. Goat anti-mouse monovalent Fab fragments.
7. PCR thermocycler.
8. Solulink Bioconjugation s-HyNic modification kit (Solulink).
9. Vivaspin 6–3,000 g/mol molecular weight (MW) Centrifugal Concentrator (Sartorius).
10. Zeba Spin Desalting columns (Thermo Scientific).

2.2 Oligonucleotide Barcode Array Components

1. Oligonucleotide barcode print solution: 25 mL of 50 mM sodium phosphate buffer (pH 8.5), 0.001 % sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS) without Ca²⁺/Mg²⁺. Prepare fresh.
2. Sonication wash buffer: 525 mL of 0.1 % SDS, 1 mM EDTA. Prepare fresh.
3. Slide H blocking solution: 25 mL of 50 mM ethanolamine, 50 mM sodium tetraborate buffer (pH 9.0).
4. Nexterion® Slide H slides (Schott, catalog number 1070936).
5. Stealth Solid Microarray Printing Pins with 0.015" diameter tips (Arrayit, catalog number SSP015).
6. Bio-Rad VersArray Compact Microarrayer (Bio-Rad, Hercules, CA).
7. Bio-Rad VersArray Printing Software (Bio-Rad, Hercules, CA).

Table 1
List of primary antibodies and isotype controls with their respective oligonucleotide-Fab fragment and complementary array sequences

Tag	Antibody	Isotype	Array sequence	Template sequence
99	CD3	IgG2a	5'-TTCAACCTCATCCGAGTGGCTCCAATAGGA-3'	5'-AAAAAAAAAAATCAACCTCATCCGAGTGGCTCCAATAGGACCCCTATAGTGAGTCGTATTAGGAATTCAT-3'
9802	CD86	IgG2b	5'-CTTCGGGGAGTGGCATCTAAAGTAGACTGA-3'	5'-AAAAAAAAAACTTCGGGGAGTGGCATCTAAAGTAGACTGATAGTCTGATAGGAAATTCAT-3'
9640	CD18	IgG1	5'-GAAAGTATTCCCTCGAGGGGATCAGCGTGATA-3'	5'-AAAAAAAAAAAGAAATTCCTCGAGGGGATCAGCGTGATACCCCTATAGTCTGATAGGAAATTCAT-3'
9494	CD44	IgG2b	5'-GTGGTTTGCCTAATGCCAGAAATGACCCGCA-3'	5'-AAAAAAAAAAAGTGGTTTGCCTAATGCCAGAAATGACCCGACCCCTATAGTCTGATAGGAAATTCAT-3'
9207	CD25	IgG1	5'-AGCGGATACTATGCCCTTCTGAGCAGCTCAA-3'	5'-AAAAAAAAAAAGCGGATACTATGCCCTTCTGAGCAGCTCAAACCCCTATAGTCTGATAGGAAATTCAT-3'
9182	CD45	IgG1	5'-AGCACAGGAGTTACTAGCTAAAGCGTTTCC-3'	5'-AAAAAAAAAAAGCACAGGAGTTACTAGCTAAAGCGGTTTCCCCCTATAGTCTGATAGGAAATTCAT-3'
9165	CD9	IgG1	5'-ATGCAGTACAAGGACAACGGGTCGGTCTTT-3'	5'-AAAAAAAAAAATGCAGTACAAGGACAACGGGTCGGTCTTTCCCTATAGTCTGATAGGAAATTCAT-3'
8552	A1TR/G1TR	IgG1	5'-GCCGTAGCCAGGGCCCTGTAGAAAATATTGT-3'	5'-AAAAAAAAAAAGCCGTAGCCAGGGCCCTGTAGAAAATATTGTCCCTATAGTCTGATAGGAAATTCAT-3'
8430	CD11c	IgG1	5'-AGACCGCTCAGGGTTGGGTGCATTAGAATAC-3'	5'-AAAAAAAAAAAGACCGCTCAGGGTTGGGTGCATTAGAATACCCCTATAGTCTGATAGGAAATTCAT-3'
8226	HLA-DR	IgG2b	5'-TCGGGAAACCGGAGTCAATGTACAACCTTGTG-3'	5'-AAAAAAAAAAATCGGGAAACCGGAGTCAATGTACAACCTTTGCCCTATAGTCTGATAGGAAATTCAT-3'
8122	CD1a	IgG1	5'-CGCGTTGCAAAGGGACCCGTTTACCATTAA-3'	5'-AAAAAAAAAAACCGGTTTGCAAAAGGGACCCGTTTACCATTAAACCCCTATAGTCTGATAGGAAATTCAT-3'
7794	CD38	IgG1	5'-TCTACTCAAGCAGACTGAGACGTTTGGG-3'	5'-AAAAAAAAAATCTACTCAAGCAGACTGAGACGTTTGGGCCCTATAGTCTGATAGGAAATTCAT-3'

7553	CD34	IgG1	5'-GTCAGTTTTCGGCCGTAGCTAATGAAGCAGA-3'	5'-AAAAAAAAAAAGTCAGTTTCGGCCGTAGCTAATGAAGCAGACCCCTATAGTGAGTCGTAATAGGAATCCCAT-3'
7425	CD11b	IgG1	5'-GCTGATTTTCAGTATGGCCAGAGATAGCCA-3'	5'-AAAAAAAAAAAGCTGATTTTCAGTATGGCCAGAGATA GCCACCCCTATAGTGAGTCGTAATAGGAATCCCAT-3'
7130	CXCR4	IgG2a	5'-ATGGCCGAGAATGCGAAGCTTCCCTATGTAG-3'	5'-AAAAAAAAAAATGGCAGAAATGCGAAAGCTTCCCTATGTAGCCCTATAGTGAGTCGTAATAGGAATCCCAT-3'
6991	CD45RB	IgG1	5'-AGGCTCGATGATTTACACAGAGCATTGGCC-3'	5'-AAAAAAAAAAAGGCTCGATGATTTACACAGAGCATTGGCCCCCTATAGTGAGTCGTAATAGGAATCCCAT-3'
6861	CD62L	IgG1	5'-TACACGATACCAGATTCATAGGTTGCCGGC-3'	5'-AAAAAAAAAAATACACGATACCAGATTCATAGGTTGCCCGCCCCCTATAGTGAGTCGTAATAGGAATCCCAT-3'
6491	CD95	IgG1	5'-TCAGTTTAACTAGCAGTCCGTCCGGCAAGAC-3'	5'-AAAAAAAAAAATCAGTTTAACTAGCAGTCCGTCCGGCAAGACAGACCCCTATAGTGAGTCGTAATAGGAATCCCAT-3'
641	CD11a	IgG1	5'-TTAAGTGTCTACAACCTGACCACCACCTCCG-3'	5'-AAAAAAAAAAATTAAGTGTCTACAACCTGACCACCACCTCCGCCCTATAGTGAGTCGTAATAGGAATCCCAT-3'
5896	CD154	IgG1	5'-TGAACCTGGCGATAGATGGCACGTTGAG-3'	5'-AAAAAAAAAAATGAACCTGGCGATAGATGGCACGTTGAGCTGAGCCCTATAGTGAGTCGTAATAGGAATCCCAT-3'
5891	CD117	IgG1	5'-GTGATATAAATCGGCCCCACATTTCCGACGG-3'	5'-AAAAAAAAAAAGTGATATAAATCGGCCCCACATTTCCGACAGCCCTATAGTGAGTCGTAATAGGAATCCCAT-3'
5757	TWEAK	IgG2a	5'-CCAGAGGCATTTCCGGAAACACTGCTGTAATT-3'	5'-AAAAAAAAAAACCCAGAGGCATTTCCGGAAACACTGCTGTAATTTCCCTATAGTGAGTCGTAATAGGAATCCCAT-3'
5509	CD2	IgG1	5'-CTCGAAACCAACAACCTGTGTGGGATTCATG-3'	5'-AAAAAAAAAAACTCGAAACCAACAACCTGTGTGGGATTCATGCTGCCCTATAGTGAGTCGTAATAGGAATCCCAT-3'
5104	CD43	IgG1	5'-TACCTATCAGAACAGATTTGGCTGGCCGCTA-3'	5'-AAAAAAAAAAATACCTATCAGAACAGATTTGGCTGGCCGCTGCTACCCCTATAGTGAGTCGTAATAGGAATCCCAT-3'
5062	CD16	IgG1	5'-TGCCTTATCAGAGCCCTAACCCCAATTAGC-3'	5'-AAAAAAAAAAATGCTTATCAGAGCCCTAACCCCAATTAGCCCTATAGTGAGTCGTAATAGGAATCCCAT-3'

(continued)

Table 1
(continued)

Tag	Antibody	Isotype	Array sequence	Template sequence
4820	CD45RO	IgG2a	5'-CAGGGATAATTCTCCAGGTCATCACTGAG-3'	5'-AAAAAAAAAACAGGGATAAATCTCCAGGTCATCAC TGAGCCCTATAGTGAGTCGTATTAGGAATCCAT-3'
4810	CD124	IgG1	5'-CCTATGGACAGTCGGTAAAGCTACCCTGT-3'	5'-AAAAAAAAAACCTATGGACAGTCGGTAAAGCTACC CTGTCCCTATAGTGAGTCGTATTAGGAATCCAT-3'
4227	CD180	IgG1	5'-ACGTCAATTATAGGCAGGCTGGATCAACTCC-3'	5'-AAAAAAAAAACGTCATTTATAGGCAGGCTGGATCAA CTCCCCCTATAGTGAGTCGTATTAGGAATCCAT-3'
3381	CD29	IgG1	5'-ATTCCCGCCAGGTGACAGTTTGCACTAAGA-3'	5'-AAAAAAAAAAATTTCCCGCCAGGTGACAGTTTGCACT AAGACCCCTATAGTGAGTCGTATTAGGAATCCAT-3'
3218	CD40	IgG1	5'-TGAATTACCCACACTAGGAGTCGGTAGTCG-3'	5'-AAAAAAAAAAATGAATTACCCACACTAGGAGTCGGTA GTCGCCCTATAGTGAGTCGTATTAGGAATCCAT-3'
3171	CD54	IgG1	5'-GAAAGATGTTGTGCGAAATGTCCAGCCTGG-3'	5'-AAAAAAAAAAGAAAGATGTTGTGCGAAATGTCCAGC CTGGCCCTATAGTGAGTCGTATTAGGAATCCAT-3'
2233	CD28	IgG1	5'-GGAAAATTTTCAGCCCCATGGGATGGACGT-3'	5'-AAAAAAAAAAGAAAATTTTCAGCCCCATGGGATGG ACGTCCCTATAGTGAGTCGTATTAGGAATCCAT-3'
2186	TLR2	IgG1	5'-ATGCCCGGCCACTACTTGTGGTCGAGGGC-3'	5'-AAAAAAAAAAATGCCCGGCCACTACTTGTGGTCGA GGGCCCTATAGTGAGTCGTATTAGGAATCCAT-3'
1698	CD49d	IgG1	5'-GCTATGGACCGCGGCAATTTATGAGAAC-3'	5'-AAAAAAAAAAGCTATGGACCGCGGCAATTTATGA GAACCCCTATAGTGAGTCGTATTAGGAATCCAT-3'
1606	TRAIL	IgG1	5'-ATGTGAAAGAGTGTTCAGCTCGACGGACTAC-3'	5'-AAAAAAAAAAATGTGAAAGAGTGTTCAGCTCGACGGA CTACCCCTATAGTGAGTCGTATTAGGAATCCAT-3'
1064	CD45RA	IgG2b	5'-CAGAACAGATGTTTTTCGGACGTAGCTGAGC-3'	5'-AAAAAAAAAAACAGAACAGATGTTTTTCGGACGTAGCT GAGGCCCTATAGTGAGTCGTATTAGGAATCCAT-3'

For microarray printing, array sequences were synthesized with a 5' primary amine and six-carbon spacer

2.3 Mast Cell Stimulation Components

1. HMC-1 media: IMDM, 10 % fetal calf serum (FCS), 150 μ M monothioglycerol, 100 IU/mL penicillin, 100 μ g/mL streptomycin, 2 mM L-glutamine. Store at 4 °C.
2. Lipopolysaccharide (LPS) stock: 10 μ g/mL LPS in sterile H₂O. Aliquot and store at -20 °C.
3. Phorbol 12-myristate 13-acetate (PMA) stock: 5 mg/mL PMA in dimethyl sulfoxide (DMSO). Aliquot and store at -20 °C.
4. Ionomycin calcium salt stock: 1 mM ionomycin in DMSO. Aliquot and store at -20 °C.

2.4 HIT Cell Processing Components

1. HIT buffer: 500 mL of 15 mM EDTA, 1.5 % bovine serum albumin (BSA), 0.05 % NaAz in PBS without Ca²⁺/Mg²⁺. Filter with 500 mL, 0.2 μ m vacuum filter. Prepare fresh and store on ice or at 4 °C for all steps.
2. Cell fixation buffer: 0.4 % formaldehyde in PBS without Ca²⁺/Mg²⁺. Formaldehyde must be methanol free (e.g., 16 % formaldehyde; Polysciences, catalog number 18814-20). Prepare fresh.
3. 10 mg/mL mouse gamma globulin (Jackson Immunoresearch, catalog number 015-000-002) in HIT buffer.
4. Cy3- and Cy5-NTP mixtures: 2.5 mM NTP mix, 3:1 unlabeled:labeled cyanine-UTP. Add 100 μ L of 10 mM unlabeled ATP, CTP, and GTP and 75 μ L 10 mM unlabeled UTP to two 0.5 mL microfuge tubes. Add 25 μ L 10 mM Cyanine-3-UTP (Enzo Life Sciences, catalog number enz-42505) or 25 μ L 10 mM Cyanine-5-UTP (Enzo Life Sciences, catalog number enz-42506) to each tube (400 μ L total). Mix by vortexing and store at -20 °C.
5. Cy3- and Cy5-amplification mixtures: 10 U/ μ L T7 RNA polymerase (Applied Biosystems), 1 \times transcription buffer (Ambion), 0.5 U/ μ L SUPERase-In™ (Applied Biosystems), 4 U/mL yeast pyrophosphatase (NEB), 0.5 mM Cy3-NTP, or Cy5-NTP mix. Prepare 30 μ L/reaction immediately before amplification. Mix by vortexing and store at -20 °C.
6. 20 \times SSC (saline-sodium citrate) buffer: 0.3 M sodium citrate (pH 7), 3 M sodium chloride. May also be purchased commercially.
7. Hybridization mixture: 2 \times SSC, 0.1 % SDS, 0.1 % salmon sperm DNA. Prepare 53 μ L/sample. Mix by vortexing and store on ice before use. Prepare fresh.
8. Post-hybridization wash buffers (PHWBs): Make 500 mL each.
 - (a) PHWB-1: 2 \times SSC, 0.1 % SDS.
 - (b) PHWB-2: 1 \times SSC.
 - (c) PHWB-3: 0.2 \times SSC.
 - (d) PHWB-4: 0.05 \times SSC.

9. RNeasy MinElute Cleanup Kit (QIAGEN).
10. RNaseZap Decontamination Solution (Applied Biosystems).
11. UltraPure DNase/RNase-Free Distilled Water.
12. Microarray Hybridization Cassette 4X16 (Arrayit, catalog number AHC4X16).
13. 4-Well dish, non-treated, sterile with lid (Thermo Scientific, catalog number 267061).
14. 96-Well, V-bottom plates.
15. Ethanol (EtOH) (200 proof).
16. Beta-mercaptoethanol (2-ME).

2.5 Microarray Scanning and Analysis Components

1. GenePix® 4000 Scanner (Molecular Devices, Sunnyvale, CA).
2. GenePix® Pro 6.0 Software (Molecular Devices, Sunnyvale, CA).
3. MeV: MultiExperiment Viewer v4.7 software (MeV, Boston, MA).
4. Microsoft Excel (Microsoft, Redmond, WA) or equivalent software for graphing and statistical analyses.

2.6 Flow Cytometry Components

1. FACS buffer: 2 % FCS, 1 mM EDTA, 0.05 % NaAz in PBS without Ca²⁺/Mg²⁺. Mix and store at 4 °C.
2. Alexa Fluor 488 goat-anti-mouse IgG (H + L) (Invitrogen).
3. 1.2 mL FACS cluster tubes (Corning Inc., Cat#4412).
4. FACScan flow cytometer (BD, Franklin Lakes, NJ).
5. Cell Quest Pro software (BD, Franklin Lakes, NJ).

3 Methods

Carry out all procedures at room temperature (RT) unless otherwise specified.

3.1 Fab-Oligonucleotide Synthesis

1. Concentrate goat anti-mouse monovalent Fab fragments to 10 mg/mL on Vivaspin 6 Centrifugal Concentrator spin columns.
2. Modify fragments with succinimidyl 6-hydrazinonicotinate acetone hydrazine (SANH) Solulink Bioconjugation s-HyNic modification kit according to the manufacturer's protocol.
3. Remove unbound SANH using Zeba Spin Desalting columns. Perform desalt three times.
4. Generate benzaldehyde-modified double-stranded oligonucleotide tags with T7 promoter and barcode sequences as follows:
 - (a) Mix 70-mer template strands containing barcode sequences flanked between T7 promoter and polyadenylated tail

sequences with 5' benzaldehyde-modified T7 promoter sequence and 40-mer reverse complement sequence in equimolar ratio (*see* Table 1).

(b) Anneal samples with oligonucleotide annealing buffer in iCycler PCR machine by cooling from 95 °C to 4 °C, decreasing 0.5 °C every 30 s.

5. Mix aliquots of desalted hydrazine-modified Fab fragments with benzaldehyde-modified oligonucleotide tags at a molar ratio of 1:2 Fab to oligonucleotide in Fab-oligonucleotide conjugation buffer.
6. Incubate reaction for 12 h at 21–23 °C.
7. Transfer reaction to 4 °C and incubate for an additional 12 h.
8. Store conjugates in Fab-oligonucleotide storage buffer at –20 °C.

3.2 Preparation of Oligonucleotide Barcode Arrays

Print Plate Preparation

1. Resuspend 5'-amine-modified 30-mer array oligonucleotides (Table 1) to a final concentration of 50 μM in oligonucleotide barcode print solution (*see* Note 1).
2. Aliquot 12 μL of each tag per well in a 384-well plate (*see* Note 2).
3. Seal plates and store at –20 °C until ready to use (*see* Note 3).
4. When ready to print, thaw plates at RT and centrifuge at 200–300×*g* for 1 min.

Print Setup

5. Turn on VersArray Compact Microarrayer and open VersArray Printing Software on computer.
6. Click “Homing” icon to home pin printer head and load stealth solid microarray printing pins.
7. Fill sonication washbasin with 525 mL sonication wash buffer.
8. Fill water bath with 60 mL ddH₂O.
9. Set humidifier to 30–50 % humidity (*see* Note 4).
10. Go to toolbar and select “run & calibration”—modify program (specify the number of slides to print) and save file.
11. Go to toolbar, select “view” and then “console,” click “open a run,” and select program to run.
12. Click “Washing” on console to perform one wash cycle before printing (*see* Note 5).
13. Load slides and print plate #1.
14. Select “start” and run from “beginning” of program.

During Print

15. Check to make sure that printing pins dip properly in print plate wells. During wash cycles, check to make sure that pins are submerged in sonication wash buffer and ddH₂O (*see* Note 6).

16. Switch print plates when necessary.
17. Add 5 mL ddH₂O to sonication washbasin every 1–2 h if necessary.
18. Add 1 mL ddH₂O to water bath every 1–2 h if necessary.

Post-print

19. Increase humidity to 75 % and incubate slides in arrayer for an additional 2 h to immobilize DNA.
20. After immobilizing, click “Drain W1” to drain water bath.
21. Empty sonication washbasin manually.
22. Add 500 mL ddH₂O to sonication washbasin to rinse. Empty manually.
23. Remove pins and slides.
24. Turn off arrayer and computer.
25. Place slides in slide box and vacuum desiccate for at least 2 h to overnight at RT.
26. Vacuum seal slides and store at 4 °C (*see Note 7*).

3.3 Stimulation and Fixation of Human Mast Cells

1. Maintain HMC-1 cells (a kind gift from Dr. Joseph Butterfield, Mayo Clinic) between 2×10^5 and 2×10^6 cells/mL in HMC-1 media in a humidified 5 % CO₂ incubator at 37 °C. Passage cells every 3–5 days.
2. Centrifuge cells at $200 \times g$ and resuspend in fresh media at 10^6 cells/mL.
3. Stimulate cells with 1 µg/mL LPS, 50 ng/mL PMA, and 1 µM ionomycin, or stimuli of interest for 8 and 24 h (*see Note 8*). Keep unstimulated cells at each time point as controls.
4. Place cells on ice for 10 min to end stimulation. Assess cells for viability and perform live cell count (*see Note 9*).
5. Centrifuge cells at $200 \times g$. Aspirate supernatant and gently flick pellet to dislodge cells.
6. Add 10 mL cell fixation buffer and place at RT for 1 h, resuspending with pipette periodically to avoid cell clumping. If necessary, live cells can be analyzed instead (*see Note 10*).
7. Centrifuge cells at $200 \times g$. Aspirate supernatant, gently flick pellet, and add 5 mL FACS buffer. Gently pipette up and down to dislodge cell clumps. Repeat once more (*see Note 11*).
8. Resuspend fixed cells in 10 mL FACS buffer and perform cell count.
9. Keep fixed cells in FACS buffer on ice or at 4 °C (long term, ~1 week) until ready to stain.

3.4 Preparation of Staining Cocktails

1. Prepare each antibody-oligonucleotide conjugate (Table 1) in a 96-well, V-bottom cell culture plate on ice as follows: (0.2 µg \times number of samples) monoclonal antibody or isotype

control per well with (0.2 $\mu\text{g} \times \text{number of samples}$) Fab-oligonucleotide (e.g., 2 μg mAb and 2 μg Fab-oligonucleotide per well for a ten-sample experiment). This results in a 3:1 Fab:antibody molar ratio.

2. Allow conjugation to proceed for 2 h at 4 °C.
3. Add 5 μg of mouse gamma globulin per μg of Fab-oligonucleotide to each well and pipette up and down slowly to mix. Incubate for 10 min at 4 °C.
4. Quickly pool antibody-oligonucleotide conjugates together into a single 1.5 mL tube (1 mAb cocktail and 1 isotype cocktail) and dilute to a final concentration of 5 $\mu\text{g}/\text{mL}$ of each mAb with HIT buffer (*see Note 12*).
5. Keep cocktails on ice or at 4 °C until ready to stain cells.

3.5 Cell Staining

Keep cells on ice for stain.

1. Add 200 μL HIT buffer/well to a 96-well, V-bottom plate to block for 1 h.
2. Flick “blocked” 96-well plate into sink to remove HIT buffer.
3. Add cells to blocked plate ($1\text{--}3 \times 10^5$ cells/well).
4. Centrifuge plate at $200 \times g$ for 3 min.
5. Flick plate to discard supernatant. Keep cells on ice until ready to stain. If staining for intracellular antigens, permeabilize cells before staining (*see Note 13*).
6. Add 35 μL cocktail per well with either mAb or isotype cocktail and gently pipette up and down.
7. Incubate for 45 min at 4 °C.
8. Centrifuge at $200 \times g$ for 3 min. Add 200 μL ice-cold HIT buffer/well and gently pipette up and down to wash. Repeat three times (*see Note 14*).
9. Repeat **step 8** twice more with ice-cold PBS.
10. Flick plate to discard supernatant. Keep cells on ice.

Oligonucleotide Barcode Amplification

1. Add 30 μL Cy3- or Cy5-amplification mix to cells and pipette to mix.
2. Add 1 μL 1/100 dilution of mAb or isotype cocktail to 39 μL Cy3- and Cy5-amplification mixes in separate wells as positive controls.
3. Amplify samples for 2 h at 37 °C on orbital shaker with gentle agitation.

RNA Purification

RNA purification protocol adapted from Qiagen Minelute RNA purification kit.

1. Prepare RLT solution: Add 10 μL of 2ME per 1 mL RLT needed (*see Note 15*).

2. Add 140 μL RLT solution to each sample (*see Note 16*).
3. Combine Cy3 and Cy5 samples in 5 mL tubes if experiment includes dye swaps. Otherwise, transfer samples directly to tubes (*see Note 17*).
4. Add 180 μL 95 % EtOH per sample and pipette or vortex to mix.
5. Transfer samples to individual RNA Minelute spin columns.
6. Centrifuge for 15 s at $9,500 \times g$.
7. Transfer spin column to new 2 mL collection tube.
8. Add 500 μL RPE buffer to each column. Centrifuge for 15 s at $9,500 \times g$. Repeat once more.
9. Transfer spin column to new 2 mL collection tube.
10. Centrifuge for 10 min at $9,500 \times g$.
11. Transfer column to 2 mL elution tube and add 14 μL RNase-free ddH₂O to the center of column (avoid touching filter).
12. Centrifuge column for 5 min at $16,000 \times g$.
13. Store RNA on ice until ready to hybridize on array. Purification should yield 12–14 μL /sample.

Pre-hybridization Blocking

14. Place printed slides in 4-well dish, printed side up.
15. Add 10 mL of slide H blocking solution per slide (*see Note 18*).
16. Block slides for 1 h at RT with rocking.
17. Aspirate slide H blocking solution and add 10 mL ddH₂O to slide until fully submerged. Repeat once more.
18. Centrifuge slides at $200 \times g$ in a metal slide rack for 5 min to dry.
19. Wash 4X16 microarray hybridization cassette with RNaseZap and pat dry. Rinse with 95 % EtOH and dry completely. Place slides in chamber, print side up. Screw cassette tightly.

Hybridization

20. Adjust block heater to 95 °C.
21. Add 53 μL hybridization buffer to each sample and place at 95 °C for 1 min.
22. Centrifuge for 5 min at $16,000 \times g$.
23. Load 65 μL of sample to each array (*see Note 19*) and seal arrays with foil tape to prevent evaporation.
24. Place hybridization cassette in humidified chamber (*see Note 20*) and hybridize arrays overnight at 42 °C with rocking.

Post-hybridization Washing

25. Prepare post-hybridization wash buffers (PHWB-1, -2, -3, -4) in Coplin jars.
26. Aspirate each well individually and wash 1 \times with 200 μL PHWB-1 (*see Note 21*).
27. Flick cassette and add 200 μL PHWB-1 per well.

28. Remove slides from cassette and quickly transfer to slide rack submerged in PHWB-1 (avoid drying). Cover Coplin jar with foil and shake for 5 min (*see Note 22*).
29. Remove slides and place in PHWB-2. Cover with foil and shake for 5 min.
30. Repeat **step 5** in PHWB-3.
31. Repeat **step 5** in PHWB-4.
32. Remove slides and centrifuge at $200\times g$ for 5 min.
33. Place slides in slide box. Cover slide box with foil and scan immediately.

Scanning

34. Open GenePix Pro 6.1.
35. Open GenePix Scanner and place slide on platform, print side down. Close scanner.
36. Select “Hardware” Icon—select one or both excitation wavelengths (532, 635) and set “PMT Gain” to 500 to start. Set “Power” to 100 %, “Pixel size” to 10 μm , “Lines to Average” to 1, and “Focus Position” to 0 μm .
37. Click “double arrow” icon to preview image. Adjust PMT gain to obtain greatest signal:noise ratio and to ensure that spots are not saturated.
38. After selecting an optimal PMT, click “single arrow” icon to capture high-resolution image (Fig. 1a).
39. When scan is finished, click “Disk” icon and select “Save Image.” Select “multiple-image file” if scanning with both wavelengths. Choose “single-image file” and select 532 or 635 if scanning with a single wavelength.

Gridding

40. Open GenePix Pro 6.1.
41. Click “Disk” icon and select “Open Images.” Select image to grid (*see Note 23*).
42. Click “Disk” icon and select “Load Array List.” Select gal file. Grid will appear over selected image.
43. Move grids to fit arrays (*see Note 24*).
44. Click “Align Blocks” icon and select “Options.” Click “Alignment” tab. Select “Find irregular features” and “Resize feature during alignment.” Adjust “minimum diameter” to 33 % and “maximum diameter” to 300 %. Select “Estimate warping and rotation when finding blocks.” Adjust “Automated Image Registration, Max translation value” to 10.
45. Set Composite pixel intensity (CPI) to 1 and click “OK.”
46. Click “Align Blocks” icon and select “Align Features in All Blocks.”
47. Inspect each array to make sure that grids encircle features (*see Note 25*).

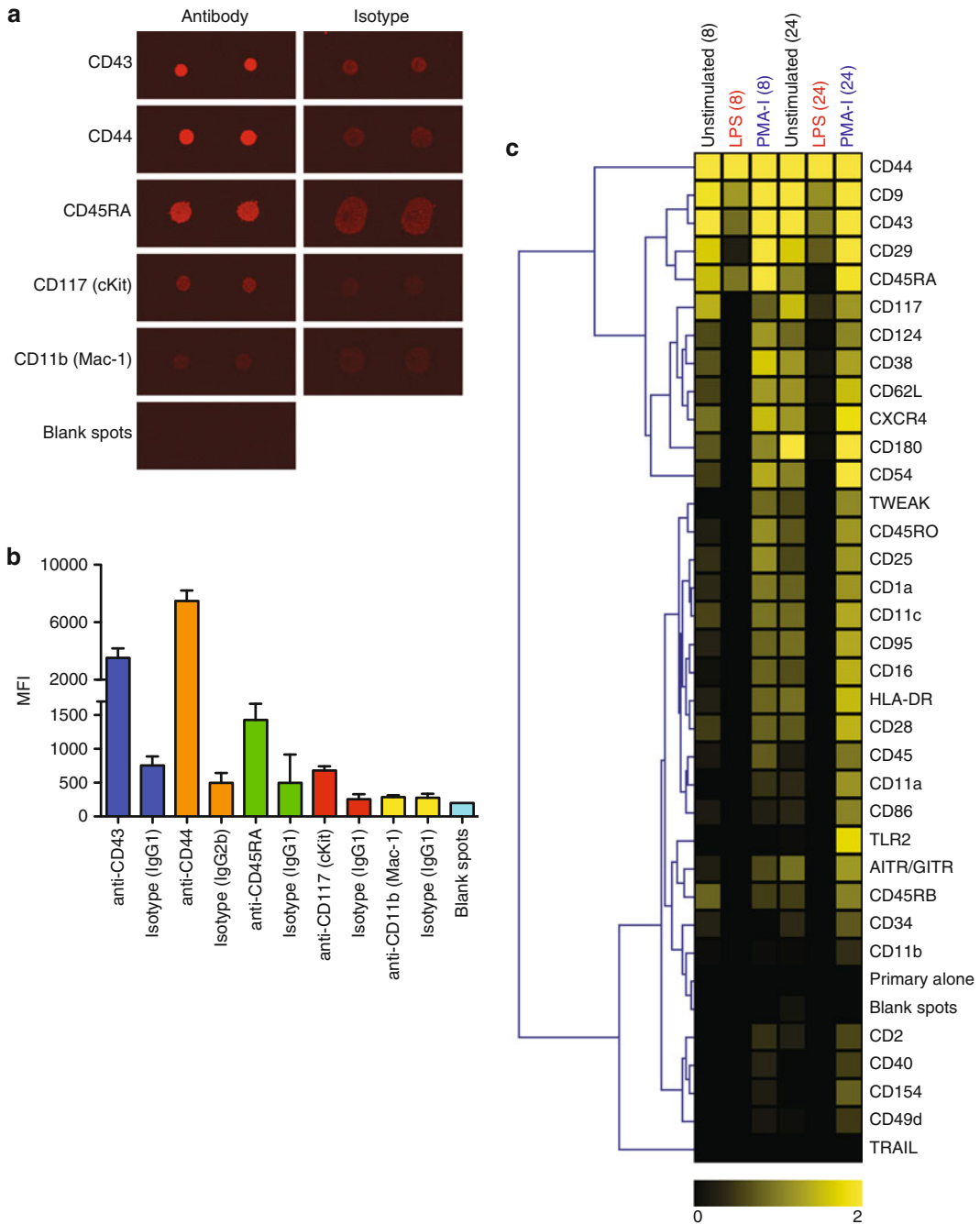


Fig. 1 Surface marker profiling of human mast cells by HIT. **(a)** Cy5 fluorescence of oligonucleotide barcode microarray stained with Cy5-labeled transcripts amplified from unstimulated HMC-1 cells stained with antibody or isotype cocktail. **(b)** Quantification of the MFI of the spots from A (bars represent SD of duplicate arrays; blank spots = barcode spots on the array for which a corresponding oligonucleotide was not included in the staining cocktail). **(c)** Unsupervised hierarchical clustering of Log₂ ratios (antibody/isotype) of 34 HMC-1 surface markers across stimulations and two time points (primary alone = cells stained with unconjugated primary antibody, to demonstrate stability of Fab/antibody complexes; length of stimulation in hours is shown in brackets)

48. Click “BCR” icon to extract data.
49. Click “Image” tab to return to image.
50. If arrays require additional flagging, click “Feature Mode” icon to highlight individual features. To flag manually, select feature and press “a.” An “X” will appear over manually flagged features (*see Note 26*).
51. To complete gridding, click “Disk” icon and select “Save Results.” Results will be saved as a GPR file.

Analysis

52. Using Excel, open the array’s GenePix GPR files and copy the F635 Median (for Cy5) or F532 Median (for Cy3) and Flags columns to a new sheet. Copy all three columns if performing a dye swap.
53. Reassign spots that were flagged as “bad” (–100) as empty. Set cells flagged as “not found” (–50), or below the baseline fluorescence (suggest 200 MFI) as 200.
54. If performing a dye swap, calculate the Log_2 Cy5/Cy3 ratio for each spot.
55. Average duplicate spots on each array and then average replicate arrays (using the reciprocal for dye-swap pairs) (Fig. 1b).
56. For single-color experiments, calculate a Log_2 ratio of antibody/isotype for each pair.
57. Export the results as a tab-delimited spreadsheet and load in MeV.
58. Perform hierarchical clustering with the following options: Gene Tree, Optimize Gene Leaf Order, Euclidean distance, and Complete linkage clustering.
59. Export an image of the heatmap (Fig. 1c).

3.6 Validation of Candidate Markers via Flow Cytometry

1. Prepare HMC-1 cells as described in Subheading 3.3 and aliquot cells into a 96-well, V-bottom plate at 10^5 cells/well.
2. Using one well per antibody, stain the cells with 50 μL of 2.5 $\mu\text{g}/\text{mL}$ primary antibody for 20 min on ice. Remember to include isotype, unstained, and secondary alone controls. Antibody staining concentrations may need to be titrated individually.
3. Centrifuge at $200\times g$, flick plate to remove supernatant, and wash with 200 μL FACS buffer per well.
4. Stain cells with a 1/1,000 dilution of fluorescently labeled anti-mouse secondary antibody for 20 min on ice. Secondary antibody dilution may need to be titrated separately.
5. Centrifuge at $200\times g$, flick plate to remove supernatant, and wash with 200 μL FACS buffer per well.
6. Resuspend in 100 μL of FACS buffer and transfer to a cluster tube.
7. Collect samples on a FACScan; typically 10,000 events per sample is sufficient for analysis (Fig. 2a, b).

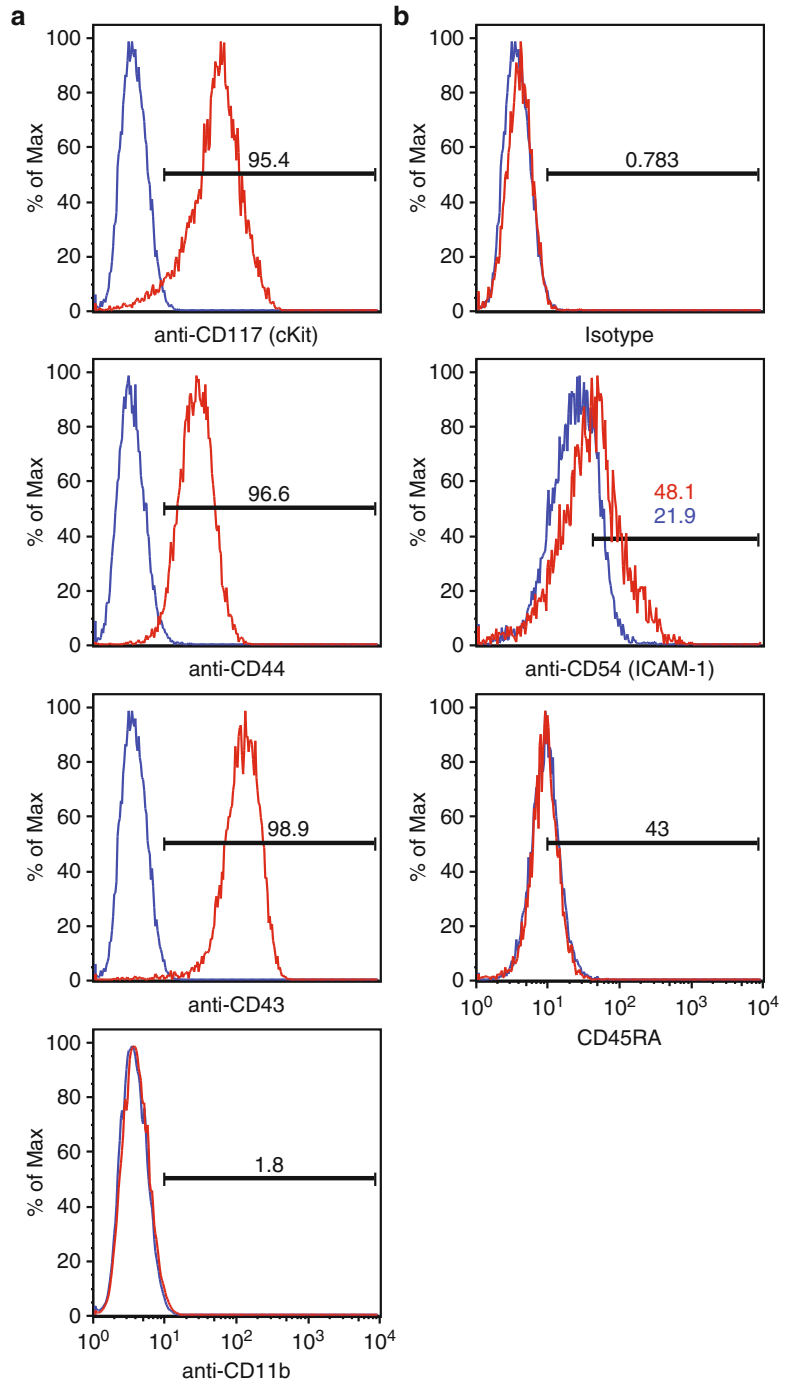


Fig. 2 HIT accurately measures mast cell surface marker levels at baseline and during stimulation. **(a)** FACS validation demonstrates that HIT correctly identified CD117 (cKit), CD44, and CD43, and the absence of CD11b on the surface of unstimulated mast cells, in agreement with previous reports (isotype = *blue line*, antibody = *red line*) [12]. **(b)** Further, FACS validation demonstrates that HIT correctly identified PMA-ionomycin stimulation-dependent upregulation of CD54 (ICAM-1), in agreement with previous reports (unstimulated = *blue line*, PMA-I = *red line*) [13].

4 Notes

1. For microarray printing, array sequences were synthesized with a 5' primary amine and six-carbon spacer.
2. Print plates can be stored for a long term (>1 year) at -20 °C. The print plate layout will need to be adapted for each specific printing setup. We recommend developing a 12-pin array printing program to print 12 arrays per slide. Before committing to a print run, perform a test print to make sure that arrays will fit microarray hybridization cassette.
3. 30 % humidity is optimal for longer print runs (>2 h).
4. Check pins to make sure that they are fully submerged in sonication buffer and wash buffer. Add more buffer if necessary. As pins hover in the vacuum platform, check to see that each pin enters a vacuum well. Pins will lift from the printer head if they are bent. After the wash cycle is complete, move printer head to door and replace pins. Repeat until all pins are flush with vacuum wells. If pins continue to lift from print head, arrayer may need to be recalibrated.
5. If an issue is encountered during a print run, immediately stop the program. Remedy the issue and modify print run program to start where it left off. Click "start" on print console and run from "middle" of program.
6. Printed slides can be stored in a sealed (airtight) slide box at 4 °C for at least 2 months.
7. Plan to stimulate more than the minimal amount of cells needed to perform experiment to accommodate cell loss during fixation.
8. Before fixing cells, it is important to assess cells for viability because significant amounts of dead cells can interfere with the assay. Remove dead cells via Ficoll gradient (GE Healthcare) or by using a MACS Dead Cell Removal Kit (Miltenyi Biotec).
9. Live cells can be analyzed; however the staining is long and traumatic. Nucleases can also decrease overall signal. If using live cells, it is critical to handle cells gently and work quickly.
10. Alternatively, postfix washes can also be performed using HIT buffer. Fixed cells can be stored in HIT buffer on ice or for a long term.

Fig. 2 (continued) This shift was not due to a change in autofluorescence, as the MFI of stimulated isotype controls and another surface marker (CD45RA) were not altered from baseline. *Horizontal bars* represent the percentage of antibody-stained cells within the gate and were set to capture ~1 % of isotype control-stained cells (except for the middle panel of **b**, where the percentages of stimulated and unstimulated are shown in *red* and *blue*, respectively)

11. We find it helpful to use a multichannel pipettor to pool tags in a single row, and then a single-channel pipettor to pool columns.
12. To prepare cells for intracellular staining, permeabilize fixed cells with 250 mL 100 % molecular grade ethanol (Sigma-Aldrich, Cat#02854) for 10 min on ice. Centrifuge at $200\times g$ and wash cells with HIT buffer. Repeat $2\times$ and proceed to stain.
13. Dye-swap experiments can be prepared as follows: unstimulated cells (Cy5) vs. stimulated cells (Cy3) on array 1, and unstimulated cells (Cy3) vs. stimulated cells (Cy5) on array 2. Alternatively, a common calibration sample can be used as follows: unstimulated cells (Cy5) vs. calibrator sample (Cy3) on array 1, and stimulated cells (Cy5) vs. calibrator sample (Cy3) on array 2. We suggest preparing aliquots of a 1/100 dilution of amplified antibody cocktail as a calibrator sample or aliquots of amplified cocktail from staining of a known cell line.
14. We find it helpful to wash cells using a multichannel pipette. To reduce cell loss, be sure to flick plate only once after each spin. Be gentle with cells and pipette up and down slowly.
15. Amount of RLT solution will vary with the number of experimental conditions. Determine the number of conditions and multiply by 0.140 to determine the total RLT (mL) required. Take RLT total and divide by 10 to determine the amount of 2ME (μL) needed to add to RLT. Mix by vortexing and store on ice.
16. Samples can be frozen in RLT solution at $-20\text{ }^{\circ}\text{C}$. Processing can continue up to a week later.
17. We find it helpful to combine/transfer samples to 1.2 mL cluster tubes (Corning Inc., Cat#4412).
18. Blocking solution is somewhat hydrophobic. If needed, pipette additional blocking solution in well to cover the entire slide.
19. It is important to avoid touching the array surface (with pipette tip, fingers, etc.) to prevent smudging of printed oligonucleotides. Be sure to switch pipette tips between loading samples to avoid cross-contamination.
20. To construct the chamber, use a small Tupperware™ container that fits the hybridization cassette and line it with damp paper towels (with PBS or ddH_2O). This step is an additional precaution to prevent evaporation.
21. To prevent drying, aspirate and wash arrays individually with a single-channel pipette before moving on to the next array. Keeping arrays hydrated will help to reduce background fluorescence while scanning.
22. It is important to keep slides covered from this point forward to prevent bleaching of the fluorescent dye.
23. GenePix scanner saves scans as Tagged Image File Format Images (TIFFs). Although GenePix 6.1 will recognize JPEG

images, we recommend gridding high-resolution TIFF images for best results.

24. There are several approaches to aligning grids. We recommend the following: Click “Block Mode” and highlight all grids. Click “Zoom Mode” and zoom into a single array. Return to “Block Mode” and align grid with features as best as possible. Click “Undo Zoom” to zoom out. Return to “Block Mode” and select first array. Use “<” and “>” keys to move across single grids. Inspect each array individually and adjust individual grid spots over array features when necessary. To do this, click “Feature Mode,” highlight spot, and move spot in position over feature.
25. If grid spot does not outline the feature after initial alignment, further adjustments can be made. If spot appears larger than a given feature, increase the CPI value and realign feature (highlight feature and select “Aligned Selected Feature”). Repeat until spot outlines feature. If GenePix does not recognize a feature despite CPI adjustment, and the feature is visible above background, spot can be adjusted manually. We suggest using this feature sparingly. In “Feature Mode” select spot. Hold down “control” and adjust spot size using “up” and “down” arrow keys.
26. Empty/Blank features flagged by GenePix appear as circles bisected by a single line. These flags are distinguishable from manual flags. To override flags (empty or manual) select spot and press “L” key. Adjust spot accordingly.

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