Supporting Information

Development of Multiplexed Bead-Based Immunoassays for Profiling Soluble Cytokines and CD163 Using Mass Cytometry

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ADDITIONAL EXPERIMENTAL DETAILS

**Materials**

Styrene (St, ≥99%), polyvinylpyrrolidone (PVP, Mw ~55 kDa), 2,2’-azobis(2-methylpropionitrile) (AIBN, 98%), Triton-X305 (TX305, 70% solution in water), diethylenetriaminepentaacetic dianhydride (DTPA dianhydride, 98%), hydrogen peroxide solution (H_2O_2, 30% in H_2O), sulfuric acid (trace metal grade), and metal salts with the purity ≥ 99.99% (trace metals basis), including lanthanum(III) chloride heptahydrate (LaCl_3·7H_2O), cerium(III) chloride heptahydrate (CeCl_3·7H_2O), praseodymium(III) acetate hydrate [Pr(OAc)_3·xH_2O], terbium(III) chloride hexahydrate (TbCl_3·6H_2O), holmium(III) chloride hexahydrate (HoCl_3·6H_2O), and thulium(III) chloride hexahydrate (TmCl_3·6H_2O) were purchased from Millipore-Sigma. 4-Vinylbenzylamine (VBA, ≥92%) was sold by TCI America. Absolute ethanol (EtOH) was manufactured by Commercial Alcohols (Mississauga, Ontario). Single-element standard solutions for inductively coupled plasma mass spectrometry (ICP-MS) calibration were purchased from PerkinElmer (Pure Plus).

**Preparation of Metal-Encoded Classifier Beads**

Metal complexes of DTPA-bis-vinylbenzyl amide (DTPA-VBAm) were employed as a chelator for incorporation different types of metal ions into polystyrene (PS) microbeads. The synthesis of the polymerizable DTPA-VBAm metal chelator and the metal loading procedure are described in our previous publication.\textsuperscript{S1} To encode La, Ce, Pr, Tb and Tm into the microbeads, we prepared La(DTPA-VBAm), Ce(DTPA-VBAm), Pr(DTPA-VBAm), Tb(DTPA-VBAm) and Tm(DTPA-VBAm) in aqueous solution by mixing DTPA-VBAm with LaCl_3, CeCl_3, Pr(OAc)_3, TbCl_3 and TmCl_3, respectively. These metal complexes were characterized by ^1H-NMR.

Microbeads as classifier beads for bead-based assays were synthesized by a series of two-stage dispersion polymerization (DisP) as described in Table S2. In a typical bead synthesis, the first stage of the polymerization of styrene (6.25 g) in absolute ethanol (18.75 g) was initiated by AIBN (0.25 g) at 70°C in the presence of PVP (1 g) and TX305 (0.35 g) as stabilizers. The reaction was protected with N_2 purging (3 mL/min) controlled by a gas mass controller (OMEGA). 2 hours after the initiation of the reaction, a warm ethanol aliquot (15 g) containing different types of metal complexes of DTPA-VBAm were added to the DisP reaction to
incorporate metal ions into microbeads. The feed of M(DTPA-VBAm$_2$) in the second-stage aliquot was optimized to produce microbeads encoded with metal ions that generate a designed level of signal intensities in MC. (see Table S3) The reaction was terminated 24 h after the initiation and cooled to room temperature. Microbeads in the reaction dispersion were purified by two sedimentation-redispersion cycles with absolute ethanol and four cycles with water. Dispersions of purified microbeads were used for scanning electronic microscopy (SEM) imaging, MC measurements and further surface modification.

The diameters and diameter distributions of microbeads were characterized from their SEM images using a Hitachi S-5200 microscope. Typically, 2 µL of a diluted bead dispersion was dropped on a 300 mesh Formvar/carbon coated copper grid and allowed to dry. The diameters of microbeads were manually measured from multiple SEM images using ImageJ software. The mean average diameter, standard deviation (SD) and the coefficient of variation (CV) were calculated based on at least 300 measurements.

In the first two trials, we explored the feed of metal complexes to the synthesis mixture to obtain microbeads that can generate MC signals with intensities in the target range. Based on the feed recipes developed in trial experiments, we then prepared 11 samples as classifier beads. These classifier beads are denoted C-1, C-2, … C-11, respectively, and are listed in Table S3.

**Acid Digestion of Microbeads**

After the termination of the DisP, an aliquot of the C-1 reaction dispersion was digested with H$_2$SO$_4$/H$_2$O$_2$ at 250°C and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) for the total metal content in the reaction. For the digestion of microbeads in the reaction dispersion, a microbead dispersion (100 µL, 0.5-2% solids content) and 500 µL of sulfuric acid were mixed in a 20 mL glass vial, heated to 250°C on a hot plate and held for 40 min with magnetic stirring, followed by the addition of a 30% H$_2$O$_2$ solution (50 µL). For ICP-MS analysis, the digestion solution was subsequently diluted with 2% HNO$_3$. To quantify the free metal content in the reaction dispersion, we filtered the reaction stock dispersion through a syringe filter (0.2 µm, Nylon) to remove the microbeads and collected the filtrate for ICP-MS analysis. We estimated the metal incorporation efficiency in the C-1 reaction by comparing the total metal content with free metal content in the reaction mixture as described in our previous publication.¹
**Instrumentation**

**Inductively coupled plasma-mass spectrometry (ICP-MS).** To quantify the metal content in samples, an ICP-MS (iCAP-Q, Thermo Scientific) system was employed. We used 2% HNO₃ to sequentially dilute the elemental standard solutions a series of concentrations of 40, 20, 10, 1, and 0.1 ppb as the calibration solutions. The metal content in each solution sample was determined based on the calibration fitting curves, with the detection limits below 10 ppt.

**ADDITIONAL RESULTS AND DISCUSSION**

**Syntheses of Metal-Encoded Classifier Beads by Dispersion Polymerization**

In this study, metal-encoded microbeads were prepared by two-stage DisP employing polymerizable metal complexes, M(DTPA-VBAm₂), to incorporate the metal ions into the PS microbeads. The synthesis of DTPA-VBAm₂ chelator was carried out in anhydrous DMSO by reacting DTPA dianhydride with 4-vinylbenzyl amine. La³⁺, Ce³⁺, Pr³⁺, Tb³⁺, Ho³⁺ and Tm³⁺ were loaded on the DTPA-VBAm₂ chelator in aqueous solution at pH 5~6. The products of these syntheses were characterized by ¹H-NMR. Details of the chelator synthesis and the metal load procedures was reported in our previous publication.¹¹ We note that an alternative method for introducing heavy metals into polymer microbeads has been reported by Mei.²²

To develop a recipe for classifier bead synthesis, our next step was to optimize the feed of M(DTPA-VBAm₂) in the second stage aliquot to prepare microbeads producing signals of ¹³⁹La, ¹⁴⁰Ce, ¹⁴¹Pr, ¹⁵⁹Tb, ¹⁶⁵Ho and ¹⁶⁹Tm in MC with intensity levels at 800~1000 counts per bead for each of these isotopic channels. Based on the linear relationship between the metal feed and metal content discovered in our previous study,¹¹ We designed the feed of six metal complexes and carried out some trial syntheses. Based on the feed recipes developed in the trial experiments, we then prepared C-1 sample as a set of classifier beads encoded with six types of metal ions.

We evaluated the metal incorporation efficiency of metal ions into C-1 beads by ICP-MS. The results shown in panel (b) of Figure S1 indicate that all six types of metal ions were effectively incorporated with efficiencies in the range of 63~74%. A slight increase in the efficiency was observed for the lanthanide metal ions with larger ionic radii, which agree with
the findings reported in our previous study. Overall, C-1 beads qualify as a candidate classifier bead in our design.

After the recipe optimization, additional types of classifier beads were prepared employing the feed of metal complexes developed for the C-1 synthesis. In the first set of samples, C-2 to C-6, two types of metal ions, Ce(DTPA-VBAm2) plus a second type of metal complex were added in the bead syntheses. The amount of each metal complex in these di-metal bead syntheses were same as in the synthesis of the C-1 beads. The same principle was then applied to the syntheses of some tri-metal-encoded microbeads (C-7 to C11) as well.

In our experience, the size of microbeads prepared by DisP varies from batch to batch, possibly due to the sensitivity of particle nucleation to the reaction conditions. To overcome this drawback of DisP and prepare microbeads with small batch-to-batch variations in particle size, we explored several control factors in the experimental conditions and found that the reproducibility of particle sizes can be improved by keeping the N2 purging and reaction heating procedures consistent across batches. As summarized in Table 1, we managed to prepare 11 types of microbeads (C-1 to C-11) that are uniform (CV < 2 %) and share a similar size with their mean diameters in the range of 2.8 to 3.0 μm.
ADDITIONAL TABLES

Table S1  Material information: cytokine standards, capture Abs, and detection biotinylated Abs used in this research

| Cytokine Analyte Standard | Capture Ab | Detection Biotinylated-Ab |
|--------------------------|------------|---------------------------|
|                          |            |                           |
| Recombinant Human Protein | Catalog #  | Clone # | Catalog # | Clone # | Catalog # |
| (carrier-free)            |            |          |            |          |            |
| IL-18/IL-1F4              | B001-5 a   | 125-2H  | D044-3 a   | 159-12B  | D045-6 a   |
| TNFα                      | 570102 b   | MAb1    | 502801 b   | MAb11    | 502903 b   |
| IL-6                      | 715104 b   | MQ2-13A5| 501101 b   | MQ2-39C3 | 501201 b   |
| IFNγ                      | 570202 b   | MD-1    | 507501 b   | 4S.B3    | 502503 b   |
| IL-4                      | 574002 b   | 8D4-8   | 500701 b   | MP4-25D2 | 500803 b   |
| CD163                     | 1607-CD a  | 215930  | MAB16071 a | 215901   | BAM16072 a |
| CXCL-9/MIG                | 392-MG a   | 49106   | MAB392 a   | Goat (poly) | BAF392 a |
| IL-10                     | 571002 b   | JES3-9D7| 501401 b   | JES3-12G8| 501502 b   |
| IL-1β                     | 579402 b   | JK1B-1  | 508201 b   | JK1B-2   | 508301 b   |

a. Vendor: R&D systems.
b. Vendor: BioLegend.

c. A desired amount of DTPA-VBAm2-metal complexes [M(DTPA-VBAm2)] dissolved in ethanol was introduced into the aliquot. Details of the metal addition are described in Table S3.

Table S2  A typical recipe for the two-stage dispersion polymerization of styrene with metal complex of DTPA-VBAm2 derivatives

| Materials (g) | 1st stage a | 2nd stage b |
|---------------|-------------|-------------|
| styrene       | 6.25        | --          |
| AIBN          | 0.25        | --          |
| PVP           | 1.00        | --          |
| TX305         | 0.35        | --          |
| ethanol       | 18.75       | 15.00       |
| DTPA-VBAm2-metal complexes | -- | c |

a. The reaction was initiated by immersing the flask in a 70°C oil bath. Prior to the initiation, the reaction solution was purged with nitrogen gas for 30 min.
b. The second-stage aliquot was introduced into the reaction mixture 2 h after the initiation.
c. A desired amount of DTPA-VBAm2-metal complexes [M(DTPA-VBAm2)] dissolved in ethanol was introduced into the aliquot. Details of the metal addition are described in Table S3.
Table S3  Summary of the feed of M(DTPA-VBAm\textsubscript{2}) in the second stage of DisP and the Abs attached to the surface of microbeads as the classifier beads

| Microbead | M(DTPA-VBAm\textsubscript{2}) Complex Feed \textsuperscript{a} (mg) |
|-----------|---------------------------------------------------------------|
| ID        | La   | Ce   | Pr   | Tb   | Ho   | Tm   |
| C-1\textsuperscript{*} | 7.6  | 8.2  | 7.6  | 3.7  | 3.3  | 3.8  |
| C-2\textsuperscript{*} | 7.6  | 8.2  | -    | -    | -    | -    |
| C-3\textsuperscript{*} | -    | 8.2  | 7.6  | -    | -    | -    |
| C-4\textsuperscript{*} | -    | 8.2  | -    | 3.7  | -    | -    |
| C-5\textsuperscript{*} | -    | 8.2  | -    | -    | 3.3  | -    |
| C-6\textsuperscript{*} | -    | 8.2  | -    | -    | -    | 3.8  |
| C-7\textsuperscript{*} | 7.6  | 8.2  | -    | 3.7  | -    | -    |
| C-8\textsuperscript{*} | -    | 8.2  | 7.6  | 3.7  | -    | -    |
| C-9\textsuperscript{*} | -    | 8.2  | -    | 3.7  | 3.3  | -    |
| C-10\textsuperscript{*} | -    | 8.2  | -    | 3.7  | -    | 3.8  |
| C-11\textsuperscript{*} | -    | 8.2  | -    | -    | 3.3  | 3.8  |

\textsuperscript{a.} Metal complexes of DTPA-VBAm\textsubscript{2} were first dissolved in 15 g of ethanol and then introduced to the DisP as the second stage aliquot.

\textsuperscript{*} The “C” in sample notation stands for “classifier”.

ADDITIONAL FIGURES

Figure S1 (a) SEM image of C-1 microbeads prepared by two-stage DisP. (b) Efficiencies of six types of metal ions incorporated into the C-1 microbeads using M(DTPA-VBAm\textsubscript{2}) complexes (M=La, Ce, Pr, Tb, Ho, and Tm). The incorporation efficiency of each metal was evaluated by comparing the free metal content after the synthesis with the total metal content in the reaction. Error bars represent the standard deviation of three measurements on the same solution.
Figure S2  Standard curves of three sets of four-plex assays for (a) IL-4, (b) IL-6, (c) IFNγ and (d) TNFα at different reporter (NP) concentrations. The x-axis in each plot represents the analyte concentration. The y-axis in each plot represents the median MC signal intensity of AuNP attached to the corresponding classifier beads. Three concentrations of AuNP with 200×, 400×, and 800× dilutions from the stock solution were investigated in these four-plex assays. Their results are presented for dilutions of 200× with blue filled circles (●), of 400× with green filled squares (■), and of 800× with red filled triangles (▲). Events with 197 Au signal intensities ≤ 1 count per bead were excluded from the statistical analysis for median intensities. The dose-response curves were drawn by fitting the experimental results with a four-parameter logistic regression model.
Figure S3  Standard curves of one set of seven-plex and two sets of eight-plex assays for (a) IL-1β, (b) IL-4, (c) IL-6, (d) IL-10, (e) IL-18, (f) IFNγ, (g) TNFα, (h) CD163, and (i) CXCL-9 for troubleshooting the background noise observed in the nine-plex assays. The x-axis in each plot represents the analyte concentration. The y-axis in each plot represents the median MC signal intensity of AuNP_{10nm} attached to the corresponding type of classifier beads. To isolate the problematic parameter in the assay, a set of seven-plex assays of standard solutions were conducted by eliminating the detection of CD163 and CXCL-9 from the system. Two sets of eight-plex assays were then carried out to individually evaluate the impact of the introduction of CD163 and CXCL-9 on the multiplex assays. In these three sets of multiplex assays, the concentrations of detection Abs were constant at 2.5 μg/mL. Negative events with ¹⁹⁷Au signal intensities of ≤ 1 count per bead were excluded from the statistical analysis for median intensities. The dose-response curves were drawn by fitting the experimental results with a four-parameter logistic regression model.
Figure S4 Summary chart of median MC signal intensities of AuNP attached to classifier beads in a series of nine-plex assays of blank samples in the absence of analyte molecules (0 pg/mL). To investigate the effect of detection Ab concentrations on non-specific binding of the Ab to classifier beads, the concentrations of biotinylated anti-CD163 and anti-CXCL9 Abs in nine-plex assays were reduced from 2.5 to 0.5 μg/mL, while the concentrations of other detection Abs remained constant at 2.5 μg/mL.

Figure S5 Standard curves of the nine-plex assays for IL-1β, IL-4, IL-6, IL-10, IL-18, IFNγ, TNFα, CD163, and CXCL-9 using the same assay conditions for the analysis of stimulated and unstimulated PBMC samples. The detection Ab concentrations of anti-CD163 and anti-CXCL9 in the assays were 0.5 μg/mL and 1.0 μg/mL, while the concentrations of other detection Abs were 2.5 μg/mL. The dose-response curves were drawn by fitting the experimental results with a four-parameter logistic regression model.
Figure S6  Cytokine concentrations in the stimulated and unstimulated PBMC samples calculated based on the dose-response standard curves in Figure S5. Some measured MC intensity values presented in Figure 5 are lower than the minimum values of the 4P-LR modeled standard curves presented in Figure S5. Therefore, no concentration can be calculated from these values. The dilution factors were taken into account in the calculation of the cytokine concentrations in the stock samples.
SUPPORTING REFERENCES

(S1) Liu, J.; Wong, E. C. N.; Lu, E.; Jarzabek, J.; Majonis, D.; Winnik, M. A. Control of Metal Content in Polystyrene Microbeads Prepared with Metal Complexes of DTPA Derivatives. *Chem. Mater.* **2021**, 33 (10), 3802–3813.

(S2) Budzinski, L.; Schulz, A. R.; Baumgart, S.; Burns, T.; Rose, T.; Hirseland, H.; Mei, H. E. Osmium-Labeled Microspheres for Bead-Based Assays in Mass Cytometry, *J Immunol* **2019**, 202:3103-3112