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Development of analytical methods for multiplex bio-assay with inductively coupled plasma mass spectrometry

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Abstract

Advances in the development of highly multiplexed bio-analytical assays with inductively coupled plasma mass spectrometry (ICP-MS) detection are discussed. Use of novel reagents specifically designed for immunological methods utilizing elemental analysis is presented. The major steps of method development, including selection of elements for tags, validation of tagged reagents, and examples of multiplexed assays, are considered in detail. The paper further describes experimental protocols for elemental tagging of antibodies, immunostaining of live and fixed human leukemia cells, and preparation of samples for ICP-MS analysis. Quantitative analysis of surface antigens on model cell lines using a cocktail of seven lanthanide labeled antibodies demonstrated high specificity and concordance with conventional immunophenotyping.

Introduction

The need for robust technology capable of capturing the large amount of information required to understand, diagnose, and cure complex human diseases is self-evident.[1,2] The development of massively multiplexed bio-analytical assays using element tags with inductively coupled plasma mass spectrometry (ICP-MS) detection has the potential to fulfil this need and is advancing rapidly.[3-12] This high information content analytical technology is posed to dramatically improve the bio-analytical toolset for research and drug discovery/validation, and consequently to benefit health care. For example, it is now recognized that simultaneous identification of multiple biomarkers with no interference between detection channels should help to fully characterize cancer:[13-17] its primary tissue source (for metastasis),[18] susceptibility to hormone treatment (breast, ovarian and thyroid cancer),[19,20] aggression and potential for invasiveness (colorectal adenoma and carcinoma).[21] Complete information will translate into personalized care, entailing higher efficacy and greatly reducing unwanted side effects. For these ambitious goals, it is particularly important to develop the best analytical methods, standards, and reference materials.

In the design of bio-analytical methods, effort is progressively shifting toward highly parallel, high throughput and highly multiplexed approaches that are able to extract large amounts of data from smaller samples with increasing efficiency. In the present work, we primarily discuss the methodology of solution analysis in view of contemporary bio-analytical work flow. Our goal is to develop instrument-independent methods which can be compared and experiments that can be performed with high reproducibility.

The first successful class of reagents for element tagging of antibodies, optimized for use with mass spectrometry, was reported recently.[22] Their utilization in solution assays is in progress, employing conventional ICP-MS instrumentation,[23] and the development of instrumentation

for single cell analysis (flow cytometry with ICP-MS detection) is highly anticipated. This paper represents a collection of immunological methods that utilize these novel reagents and elemental analysis. For anyone from the elemental analytical community planning to enter this new area of research, a steep learning curve and extensive collaboration with bio-analytical laboratories should be both expected and a requirement of a common ground for discussions.

Experimental

Selection of elements for element tags

Without lessening the general concept of element tagging, only the lanthanide group of elements will be considered in this work. It is reasonable to start from this group, taking into account that all elements in this group have similar chemical properties and the natural background is very low in typical biological samples. Except for Ce and, to a much lesser degree for Pr and Tb, the lanthanides primarily occur in the oxidation state III. Complexes of lanthanides with chelating oxygen and nitrogen ligands are the most stable. This group of ligands includes derivatives of DOTA (1,4,7,10-tetrakis(carboxymethyl)-1,4,7,10-tetraazacyclododecane) and DTPA (diethylenetriaminepentaacetic acid), known to form lanthanide chelates of high kinetic and thermodynamic stability.[24,25] These ligands were used in our study.

Table 1 gives an example of 21 isotopes for tagging based on the following assumptions: (a) ICP plasma is able to thoroughly atomize and ionize the sample (robust plasma); (b) the possible matrix (concentrated HCl, buffers, Na, K, Ca from the cell) does not affect the level of interferences; (c) all lanthanide oxides are at 3%; (d) all analytes are at the isotope abundance signal level (*e.g.*, 100% for monoisotopic) and instrumental sensitivity is equal for all isotopes; (e) other impurities have natural abundance distribution. Under these assumptions the expected level of interferences is presented in Table 1 under numbers 1, 2 and 3.

Many lanthanides can be obtained in the enriched form as oxides, for example from Trace Sciences International Inc. Impurities will also interfere with other isotopes, which can be estimated from certificates. If one selects the isotopes highlighted in Table 1, the resultant interferences will be higher, and they are presented under number 4. The total interference level is dominated by the purity of the enriched isotopes.

Additional considerations should be given to a natural dynamic range of bio-analytes. In direct analysis of a single cell, it is expected that the analyte concentrations will not exceed several millions of copies (cell surface receptors, for example) per cell. One should also not expect to measure anything below several hundred copies (a transcription factor, for example) of analyte per cell. The limit of detection is mostly determined by the non-specific binding of tagged affinity molecules to a cell. In solution assays, the dynamic range of concentrations could be significantly wider, but in this case the non-specific binding of tagged molecules to plastic and filter surfaces, as well as the specificity of affinity molecules, should be closely monitored. For these reasons, poorly enriched isotopes should not be used for dominant (highly abundant) analytes.

Experimental measurements were performed on a commercial ICP-MS instrument ELAN DRCPlus™ (PerkinElmer SCIEX) described elsewhere[26,27] and operated under the normal plasma conditions presented in Table 2. The DRC mode was not required in this work, and all measurements were carried out in the standard mode. The sample uptake rate was adjusted depending on the particular experiment and sample size, typically 100 μl min^{-1}. A Burgener Micromist nebulizer (Burgener Research, Inc) was used in all instances. Experiments were performed using an autosampler (PerkinElmer AS 91) modified for operation with Eppendorf 1.5 ml tubes in 96 sample racks. The sample size varied around 150 μl. Standards were prepared

from 1000 μg mL^{-1} PE Pure single-element standard solutions (PerkinElmer, Shelton, CT) by sequential dilution with high-purity deionized water (DIW) produced using an Elix/Gradient (Millipore, Bedford, MA) water purification system.

Selection, preparation and tagging of antibodies

Solution assay was performed on immunostained cell suspensions. Element tagged antibodies against surface and intracellular markers were used as affinity reagents. It should be clear that solution assays are not limited to this particular combination. Other examples may include: beads conjugated to antibodies for detection of specific analytes in solution; immunoprecipitation with specific antibodies from lysates; and ELISA type surface immobilized assays (96-well plate format, for example). However, the major steps in the sample preparation are very similar.

Our tagging strategy is based on using a water-soluble polymer bearing metal-chelating ligands along the backbone and a linker group.[22] The polymer contains a maleimide group at one end for coupling to free sulfhydryls generated by selective reduction of disulfide bonds in the hinge region of the immunoglobulin molecule. It is common practice to attach tags to antibodies *via*-SH groups, which is much more likely to preserve antibody activity.[28] The chelating ligand is chosen to form high-affinity complexes with lanthanide (Ln^{3+}) ions. The use of a metal-chelating polymeric tag allows us to incorporate multiple numbers of a given ion, leading to an increase in the sensitivity of the method.

Antibodies (IgG) at 0.5-1 mg ml^{-1} in phosphate buffered saline can be purchased from a wide variety of commercial sources. In this study, mouse monoclonal and rabbit polyclonal antibodies were used. The antibody must be purified and free from serum/ascites proteins or stabilizing BSA.

Antibodies were labeled with DTPA- or DOTA-containing polymer-tags (MAXPAR™ reagents, DVS Sciences Inc.) according to the following protocol. Prior to conjugation with the tag, antibodies were reduced using TCEP (tris(2-carboxyethyl)phosphine hydrochloride; Pierce) in 100 mM phosphate buffer (pH 7.2) with 2.5 mM EDTA. After 30 min incubation at 37 °C, the antibody was separated from reducing agent in a 30K Molecular Weight Cut Off centrifugal filter (Pall Nanosep), re-suspended in Tris-buffered saline (TBS, pH 7.0) at 1 mg ml^{-1} and combined with a 10-fold molar excess of polymer-tag. The antibody-tag conjugate was subsequently washed with 20 mM ammonium acetate (pH 6.0) buffer in the spin filter, combined with 5μM $LnCl_3$, and incubated for 30 min at 37 °C. TBS was used for extensive washing of the antibody at the final stage. Mouse immunoglobulins (IgG, Biomeda Inc.) were labeled with polymer-tags and the same lanthanides as the primary antibodies to serve as indicators of non-specific background binding of monoclonal antibodies to cells. Element-tagged antibodies were stored in TBS at a final concentration of 0.5 mg ml^{-1} at 4 °C.

It is very important to know the final tagged antibody concentration for quantitation and control of non-specific binding. Also, multiple steps of size exclusion filtration and washes contribute to uncertainty in the antibody concentration. In our case, immunoglobulin concentrations (native and tagged) were measured using the NanoDrop^R ND-1000 Spectrophotometer (NanoDrop Technologies Inc.) at 280 nm set for IgG. However, we expect that the tag affects the measurement in some way. This important step should be revisited later on.

Results and discussion

Validation of antibody tagging protocol

The tagging protocol needs to be validated and represents a separate challenge. During the tagging procedure it is necessary to ensure that the antibody retains specificity toward its

antigen (antigen binding sites remain intact), as well as be recognizable by anti-species specific antibodies for secondary immunoassays. First, tag conjugation was verified by performing the reaction in reducing and non-reducing conditions. Equal amounts of mouse IgG were labeled with thulium-containing tag (Tm-tag) in the presence (+) or absence (-) of TCEP. The resultant antibody conjugates were standardized to 0.58 mg ml^{-1} and serially diluted to 10, 5, 2.5, 1.25, 0.63 and 0.31 μg ml^{-1} in PBS. Similar reactions were set up for bovine serum albumin (BSA + and BSA-). To assess tag binding and structural integrity of the immunoglobulins, we used 96-well plates coated with goat anti-mouse polyclonal antibodies (Pierce Biotechnology Inc.), which are specific for the Fc fragment of mouse IgGs. The goat α-mouse antibodies are immobilized on the bottom of plate wells. For every element separately, 30μL of each dilution are transferred into wells and incubated overnight at 4 °C. If the mouse IgG conjugated to the elemental tags were denatured during labeling, the goat α-mouse antibodies would not recognize them. In the case of the 96-well plate, the washing cycle consists of repeated aspiration and the addition of 200 μL PBS per well. Unbound IgG, tag and excess metal should be washed thoroughly to reduce non-specific background. The number of washes is usually determined experimentally. Finally, 85 μL of 37% HCl (Seastar Chemicals Inc.) was added per well and incubated for 30 min. Subsequently, 80 μL from each well were transferred into Eppendorf tubes containing 80 μL of 1 ppb Ir in 10% HCl as an internal standard (160 μL total). Alternatively, without re-formatting, the standard can be added directly to the wells, and the plate analyzed by ICP-MS *via* an autosampler.

As is evident from Fig. 1, the increase in Tm concentration for IgG(+) follows a distinct saturation curve, whereas IgG(-) displays an order of magnitude lower response. This can be explained by the fact that some amount of tag non-specifically (electrostatic interaction) attaches to the immunoglobulin. On the other hand, values for BSA(+) and (-) are similar and very low, indicating that the unreacted tag and/or Tm do not comprise a significant background in the assay.

Furthermore, the influence of different metals on IgG structure and on tag loading was analyzed. In the following experiment, 13 aliquots of 50 μg of mouse IgG were labeled with the element tag containing polymer bearing DTPA metal-chelating ligands according to the previously described antibody labeling protocol. Each antibody-polymer-chelate preparation was loaded separately with 5 μL of a 0.1 M solution of Tb, Er, Lu, Tm, Ho, Eu, Pr, Dy, Yb, Nd, Gd, Sm, or Ce. Therefore, each antibody sample is labelled with a unique elemental tag independently according to the same procedure. The resultant antibody conjugate was standardized to 0.7 mg mL^{-1} and serial dilutions of 10, 5, 2.5, 1.25, 0.63, 0.31, and 0.16 μg mL^{-1} were prepared. The results of the ICP-MS analyses presented in Fig. 1 and Fig. 2 demonstrate that IgGs are not denatured after tagging. The capacity of the goat α-mouse 96 well plates is quite limited (12 pmol IgG per well, according to the manufacturer), which can be seen in Fig. 1 as a tendency towards saturation of the signal in the range of high IgG concentrations. One would expect that the results represent differences in metal uptake by the tag. Unfortunately, the exact order of effectiveness of tagging from Ce (highest) to Dy (lowest) is not very reproducible. As we noted before, this effect may be the result of deficiencies in estimating IgG concentrations, as well as losses that occur during sample preparation (washes, filtration and so on). At this stage we are unable to delineate uncertainty in the sample preparation from binding efficiency of different metals.

Demonstration of element tagged antibody reactivity on cells

Washing cell samples is a critical step in the reduction of non-specific background and variability between replicates. The cell washing cycle is time consuming but has to be done carefully, avoiding cell loss and damage. Normally, cells in suspension are distributed into Eppendorf tubes and centrifuged at low speed (300*g*) for 5 min. Liquid is carefully aspirated

from the cell pellet. The pellet is sharply flicked to break up clumps of cells, and 1 ml of fresh wash buffer (PBS) is added. The tube is briefly vortexed (shaken), and washed cells are pelleted by centrifugation for further steps.

For the analysis of intra-cellular markers fixation/permeabilization of cell samples is required. The following is one of many described methods which we found particularly useful (see Fig. 3). The washed cell pellet was re-suspended in 1% formaldehyde fixation buffer, incubated for 15 min, washed in PBS, and blocked in 100 mM glycine (10 min). Finally, cells were made permeable with ice-cold 90% methanol (10 min). After low speed centrifugation, cells were re-suspended in blocking buffer (10% serum-PBS) and incubated for 15 min.

The immuno-labeling procedure finalizes sample preparation for ICP-MS analysis (see Fig. 4). The blocked cells were counted in a hemocytometer and equal numbers (usually 10^5-10^6 cells per replicate) were distributed into triplicate tubes to which a mixture of all the element tagged antibodies was added. (We recommend using the triplicate format to reduce variability in sample preparation at the exploratory stage of research. The number of samples should be reduced for routine analysis.) Another set of triplicate tubes was incubated with a mixture of element tagged mouse IgGs as a control against the non-specific binding of antibodies to cells. Cells were incubated for 30 min for 1 h at room temperature and washed in PBS. Washed cells were stained with 1 μM Rh^{3+}-containing DNA-metallointercalator for normalization.[29,30] Finally, the washed cells were spun down, and the cellular pellets were dissolved in ultra-pure concentrated HCl. An equal volume of internal standard (1 ppb Ir in 10% HCl) was added to each tube to compensate for possible long term sensitivity drift, and samples were analyzed by ICP-MS.

Example of multiplexed assay

The following is an example of a multiplexed cellular assay using the KG-1a, acute myelogenous leukemia, and THP-1, acute monocytic leukemia, cell lines. The cell lines were purchased from ATCC, Manassas,VA. Cells were grown in alpha-MEM, supplemented with 10% FBS (HyClone) and 2 mM L-glutamine (Invitrogen), in a humidified incubator at 37 °C and 5% CO_2. Cells were split every 3-4 days. Monoclonal antibodies to surface antigens (CD33, CD34, CD38, CD45, CD64, HLA-DR) were purchased from BD Biosciences, San Jose, CA. Mouse immunoglobulins were from Biomeda Inc.

The two cell lines (KG1a and THP-1) represent different types of acute human myeloid leukemia and are characterized by the expression of specific surface markers. Seven cell surface antigens were detected simultaneously using specific antibodies labeled with element tag loaded with isotope enriched lanthanides (CD33-^{141}Pr, CD34-^{169}Tm, CD38-^{165}Ho, CD45-^{159}Tb, CD64-^{153}Eu, CD44-^{151}Eu, HLA-DR-^{147}Sm). Live suspension growing cells were collected by low speed centrifugation and washed in phosphate buffered saline. Equal cell numbers were distributed into triplicate tubes (10^5 cells per tube). All seven lanthanide tagged antibodies were mixed into one tube at approximately 1 μg ml^{-1} each. To control for non-specific immunoglobulin binding to live cells, we used mouse IgGs prepared simultaneously with specific antibodies and labeled with the same lanthanide isotopes. The cells were incubated with either antibodies or IgGs for 30 min at room temperature, then washed several times with PBS by low speed centrifugation. Cells were post-fixed in 1% formaldehyde-PBS for staining with a Rh^{3+}-containing DNA-metallointercalator for cell number normalization. Finally, the cellular pellets were dissolved in concentrated HCl, and the solution was analyzed by ICP-MS (see Fig. 5).

The less differentiated hematopoietic progenitor cell line KG1a is known to express high levels of hematopoietic precursor marker (CD34), hyaluronic acid receptor (CD44) and the leukocyte common antigen (CD45), and very low levels of myeloid precursor marker (CD33), monocyte

marker (CD64) and the human leukocyte antigen HLA-DR.[31-35] On the other hand, THP-1 cells display characteristics of more differentiated monocytes being positive for CD33, CD64, CD38, CD45, as well as HLA-DR (low) and negative for CD34.[36-38] The results, presented as a polar diagram in Fig. 5, clearly demonstrate the differences in surface marker expression between KG1a and THP-1, and are consistent with the above referenced fluorescent flow cytometry analyses.

Conclusions

Recently, our group had successfully developed tagging reagents specifically designed for analytical methods utilizing elemental analysis.[22] In this work, capitalizing on the well established analytical advantages of the ICP-MS technique, we demonstrated the typical workflow of method development for element-tagged immunoassays. There is no principal limitation for multiplicity of the antigen detection except the number of available stable isotopes. In this work we demonstrated the method on live and fixed acute leukemia cell lines which were successfully used as an example of multiplexed immunostaining. The novel methodology described includes all of the major steps, from the selection of elements for tags, validation of tagged reagents and experimental protocols for labeling antibodies, to the preparation of samples for ICP-MS analysis.

Acknowledgements

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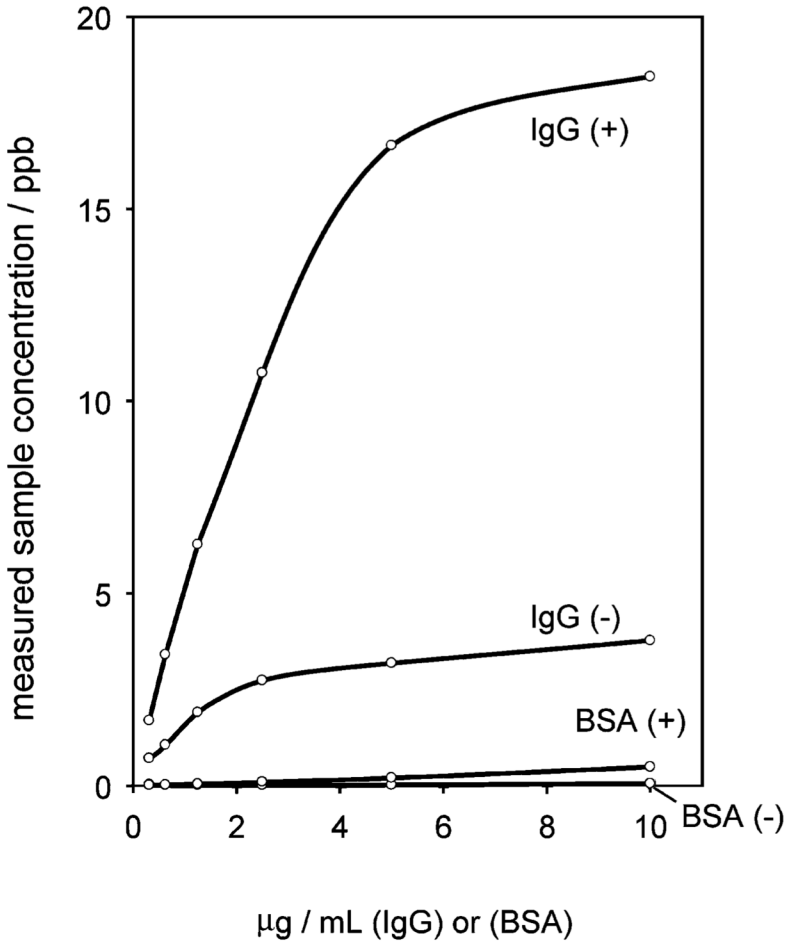

Fig. 1.
Validation of antibody conjugation using Tm-containing tag. BSA is used as a non-specific background control. (+) and (-) marks indicate the presence and absence of reducing reagent, respectively.

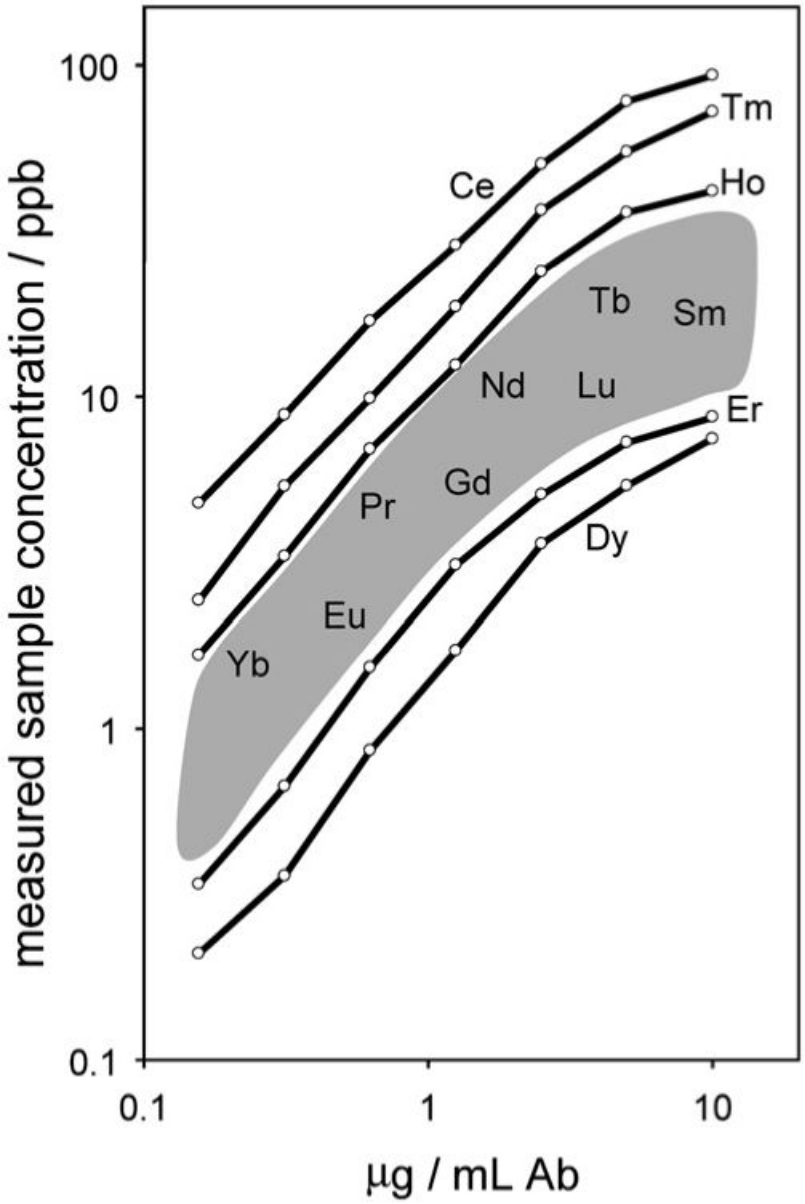

Fig. 2.

Results of multiple tagging of IgG. Every element tagged IgG was prepared and analyzed independently. Only Dy, Er, Ho, Tm, and Ce containing IgG are presented explicitly. The data from other tagged IgGs are all closely packed in the highlighted area.

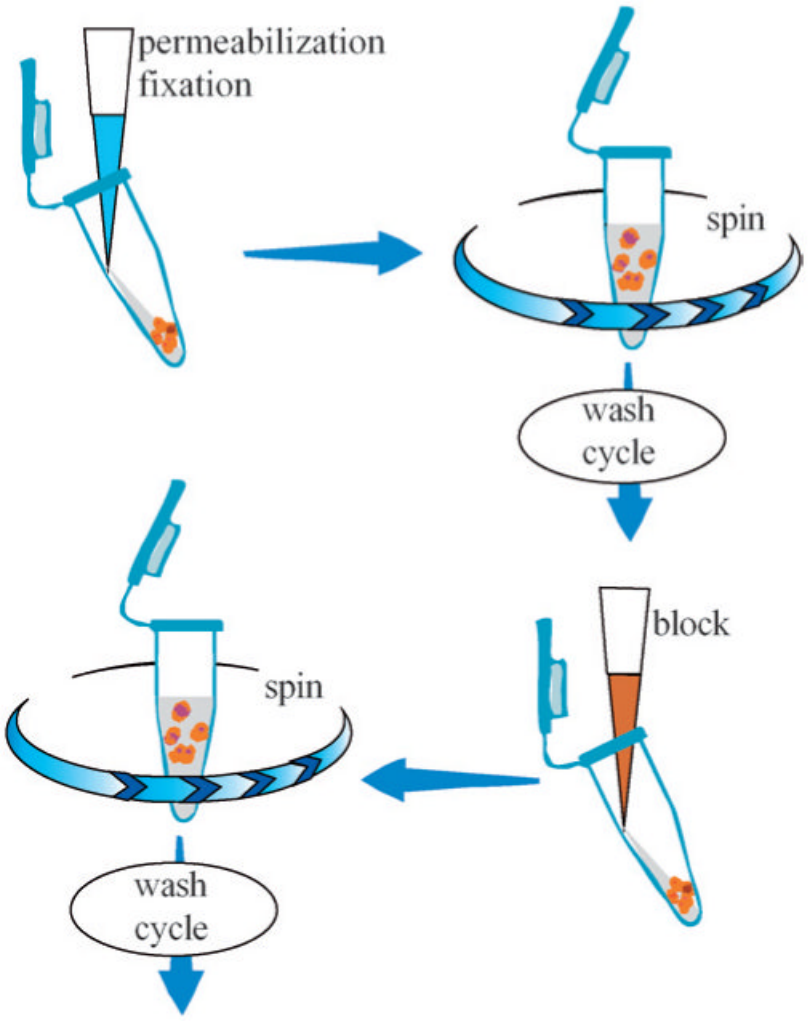

Fig. 3.

Preparation of cells for analysis of intra-cellular markers. The washed cell pellet was re-suspended in 1% formaldehyde fixation buffer, incubated for 15 min, washed in PBS, and blocked in 100 mM glycine (10 min). After additional washing, cells were made permeable with ice-cold 90% methanol (10 min) (not shown here). After low speed centrifugation, cells were re-suspended in blocking buffer (10% serum-PBS) and incubated for 15 min.

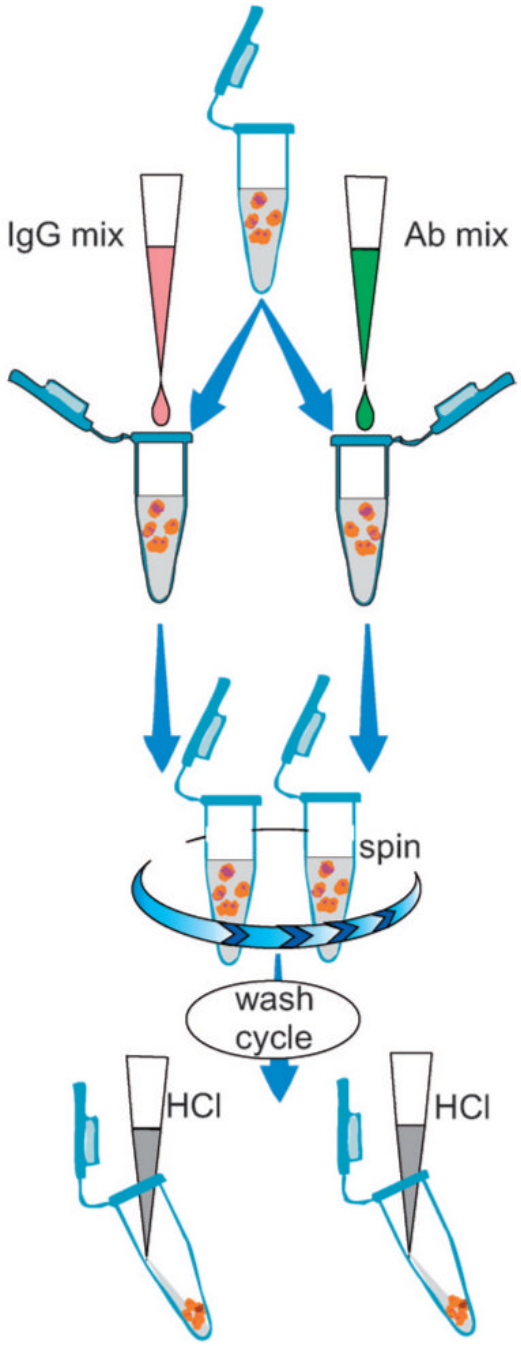

Fig. 4.

Preparation of cellular samples for ICP-MS analysis. The blocked cells were distributed into triplicate tubes to which a mixture of all the element tagged antibodies was added. Another set of triplicate tubes was incubated with a mixture of element tagged mouse IgGs as a control against the non-specific binding of antibodies to cells. Cells were incubated for 30 min for 1 h at room temperature and washed in PBS. Finally, the washed cells were spun down, and the cellular pellets were dissolved in ultra-pure concentrated HCl.

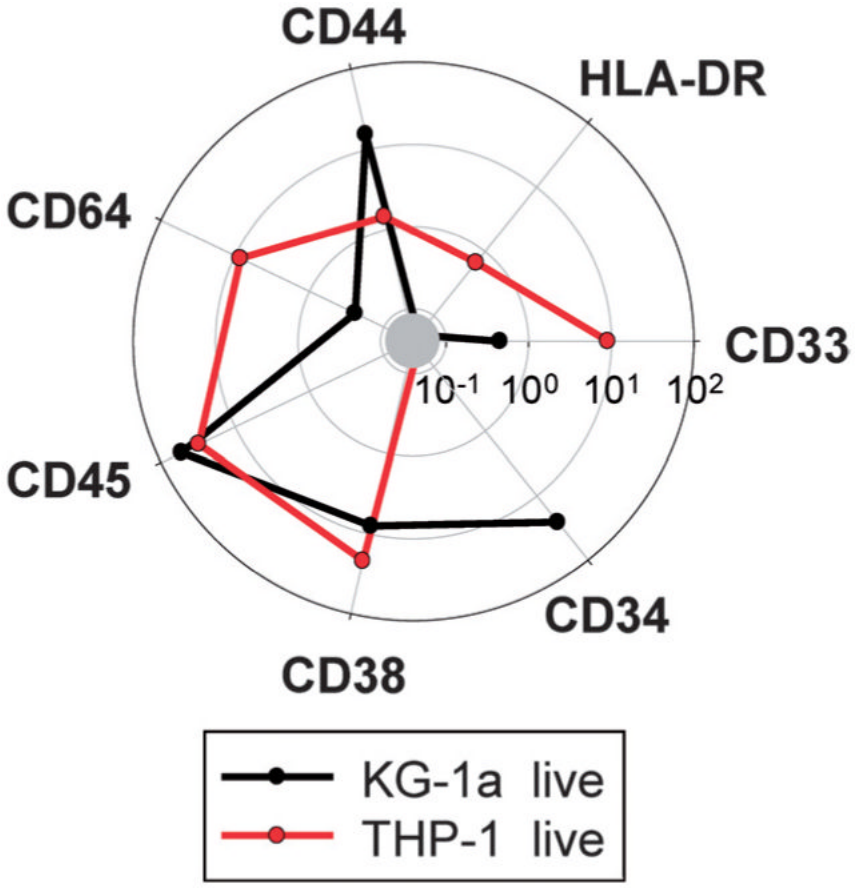

Fig. 5.

Cellular assay of KG-1a and THP-1 cell lines by ICP-MS analysis. Normalized response on selected antigens. The detector signal is normalized to the detector signal of the internal standard 1ppb of Ir (instrument sensitivity) and Rh metallointercalator (cell number). Tagged antibodies: CD33-^{141}Pr, CD34-^{169}Tm, CD38-^{165}Ho, CD45-^{159}Tb, CD64-^{153}Eu, CD44-^{151}Eu, HLA-DR-^{147}Sm. Connecting lines are plotted for clarity and do not have analytical significance, except for recognition of the expression patterns. Notice that HLA-DR-^{147}Sm was not detected for KG-1a cells and CD34-^{169}Tm was not detected for THP-1 cells.

Table 1

Interferences #1-3 are produced from naturally abundant isotopes and oxides which are assumed to be at 3%. Interference #4 is the combined certified level of elemental impurities in isotope-enriched products from Trace Sciences International Inc., Delaware. Entries in bold indicate suggested isotopes for the 21-plex assay

Isotope	Interference #1	Interference #1 (%)	Interference #2	Interference #2 (%)	Interference #3	Interference #3 (%)	Interference #4 (%)	Total (%)
^{138}Ce							0.02	0.02
^{139}La							**0.03**	**0.03**
^{140}Ce[a]							**0.00**	**0.00**
^{141}Pr							**0.12**	**0.12**
^{142}Nd	**^{142}Ce**	**0.35**					**0.66**	**1.02**
^{143}Nd[b]							1.06	1.06
^{144}Nd	**^{144}Sm**	**0.11**					**1.84**	**1.95**
^{145}Nd							**0.87**	**0.87**
^{146}Nd[c]							**4.12**	**4.12**
^{147}Sm							**0.07**	**0.07**
^{148}Sm	^{148}Nd	0.45					4.02	4.47
^{149}Sm[d]							1.11	1.11
^{150}Sm[e]	^{150}Nd	0.20					0.46	0.66
^{151}Eu	**^{135}BaO**						**0.24**	**0.24**
^{152}Sm	**^{152}Gd**	**0.01**					**0.47**	**0.48**
^{153}Eu							**0.84**	**0.84**
^{154}Sm[f]	^{154}Gd	0.05					1.09	1.14
^{155}Gd	^{139}LaO	3.00					0.90	3.90
^{156}Gd[g]	**^{156}Dy**	**0.01**	**^{140}CeO**	**2.99**			**0.05**	**3.04**
^{157}Gd	^{141}PrO	3.00					4.41	7.42
^{158}Gd	^{158}Dy	0.01	^{142}NdO	2.99	^{142}CeO	0.01	1.13	4.15
^{159}Tb	**^{143}NdO**	**0.03**					**0.24**	**0.27**
^{160}Gd	^{160}Dy	0.02	^{144}NdO	3.02			0.37	3.40
^{161}Dy	^{145}NdO	2.86					0.14	3.00
^{162}Dy	^{162}Er	0.02	^{146}NdO3.09			0.29	3.40	
^{163}Dy	^{147}SmO	2.82					1.15	3.97
^{164}Dy	**^{164}Er**	**0.03**	**^{148}NdO**	**0.01**	**^{148}SmO**	**0.12**	**0.08**	**0.24**
^{165}Ho	**^{149}SmO**	**0.03**					**0.16**	**0.19**
^{166}Er	**^{150}NdO**	**0.01**	**^{150}SmO**	**0.01**			**0.55**	**0.57**
^{167}Er	^{151}EuO	2.98					1.74	4.72
^{168}Er	^{168}Yb	0.03	^{152}SmO	2.98			1.35	4.35
^{169}Tm	**^{153}EuO**	**3.02**					**0.07**	**3.09**
^{170}Er			**^{154}SmO**	**0.03**			**0.11**	**0.14**
^{171}Yb	**^{155}GdO**	**0.03**					**0.10**	**0.13**
^{172}Yb	^{156}GdO	2.80					3.92	6.72
^{173}Yb	^{157}GdO	0.13					0.73	0.87
^{174}Yb	**^{158}GdO**	**0.03**					**2.34**	**2.37**
^{175}Lu	^{159}TbO	3.01					0.14	3.15
^{176}Yb							**0.48**	**0.49**

[a] Oxide limits usage of ^{156}Gd.

[b] Oxide interferes with monoisotopic Tb.

[c] Interferes by poor enrichment of ^{145}Nd.

[d] Oxide interferes with ^{165}Ho.

[e] Oxide interferes with ^{166}Er.

[f] Oxide interferes with ^{170}Er.

[g] Interferes mostly by ^{140}CeO.

Table 2

Typical operating conditions of the ICP-MS instrument ELAN DRCPlus™ (PerkinElmer SCIEX). The instrument was optimized to provide standard operating conditions

Parameter	Value
Plasma power	1400 W
Burgener Micromist nebulizer, Ar flow	0.95 L min^{-1}
Plasma gas, Ar flow	17 L min^{-1}
Auxiliary gas, Ar flow	1.2 L min^{-1}
CeO^+/Ce^+ ratio in 10% HCl	<3%
Typical sensitivity	
1 ppb Ir in 10% HCl, typical	10^4 cps
1 ppb In in 10% HCl, typical	4×10^4 cps