Messenger RNA Detection in Leukemia Cell lines by Novel Metal-Tagged *in situ* Hybridization using Inductively Coupled Plasma Mass Spectrometry

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Abstract: Conventional gene expression profiling relies on using fluorescent detection of hybridized probes. Physical characteristics of fluorophores impose limitations on achieving a highly multiplex gene analysis of single cells. Our work demonstrates the feasibility of using metal-tagged *in situ* hybridization for mRNA detection by inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS as an analytical detector has a number of unique and relevant properties: 1) metals and their stable isotopes generate non-overlapping distinct signals that can be detected simultaneously; 2) these signals can be measured over a wide dynamic range; 3) ICP-MS is quantitative and very sensitive. We used commercial antibodies conjugated to europium (Eu) and gold together with biotinylated oligonucleotide probes reacted with terbium-labeled streptavidin to demonstrate simultaneous mRNA and protein detection by ICP-MS in leukemia cells.

Keywords: *in situ* hybridization; oligonucleotide probes; leukemia cell lines; mass spectrometry; metal-tagged affinity reagents; multiplex gene analysis.

Abbreviations: ICP-MS, inductively coupled plasma mass spectrometry; anti-mouse-Au antibody, gold-labeled anti-mouse antibody; anti-rabbit-Eu, europium-labeled anti-rabbit antibody; StrAv-Tb, terbium-labeled streptavidin; StrAv-PerCp, streptavidin labeled with peridinin chlorohyll-a protein.

Introduction

The Human Genome project has produced a wealth of genetic sequence information that will help to diagnose and treat many human diseases. Multiple genomic technologies for simultaneous monitoring of the expression of thousands of genes have been developed, including DNA microarrays, differential display, and serial analysis of gene expression (SAGE). However, effective gene profiling for medical applications will require fine-tuning of existing methods and the introduction of new, sensitive, and robust technologies. The basic principle of all array-based methods is the hybridization of fluorescent or biotin labeled cRNA or cDNA species to oligonucleotides or complementary DNA molecules attached to solid supports. Presently, gene chip array on a glass slide or in bead format with fluorescence detection are the predominant platforms used (Churchill, 2002; Lipshutz and Fodor et al. 1999; Guo and Guilfoyle et al. 1994; Han and Gao et al. 2001; Lockhart and Chee et al. ; Pease and Solas et al. 1994). However, all fluorochrome-based analytical methods are limited by the mismatch of excitation and absorption wavelengths and the overlap of emission signals, which are physical characteristics of all fluorophores. More significantly, the emission spectra are not well baseline-resolved. This results in overlap of the fluorescent signals that becomes important when the target copy-count per cell differs substantially (a large signal in one channel overlaps strongly into the adjacent weaker signal channel) and fluorescence compensation becomes an obligate step in most FACS analyses (Herzenberg and Tung et al. 2006). Although it is feasible to have an optical emission detector capable of distinguishing more than 15 wavelengths (Carson and Vignali, 1999) and an excitation system with significantly broad range, it remains impossible to create a set of non-interfering fluorophores for massively multiplexed assays. These physical limitations represent a critical barrier to progress in the field of fluorescent multiplexed assays and are difficult to circumvent.

Nevertheless, several methods for multiplexed fluorescence detection of nucleic acids in single cells do exist, although quantitation with massive multiplexing has not been achieved. Semi-quantitative *in situ* hybridization histochemistry (ISH) is a technique used to detect the presence and estimate the

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relative abundance of specific RNA sequences in a single cell. The visualization of signal is usually achieved by chromogenic substrates or fluorochrome dyes (Carson and Vignali, 1999; Derradji and Bekaert et al. 2005; Eberwine and Yeh et al. 1992; Hakala and Lonnberg, 1997; Kadkol and Gage et al. 1999; Kellar and Iannone, 2002; Kwok and Chen ; Pease and Solas et al. 1994; Raap, 1998; Tanke and Wiegant et al. 1999; Tanke and Dirks et al. 2005; Weier and Chu et al. 2004) and is not readily amenable to multiplexing. Cytogeneticists have also developed a unique chromosome characterization method termed fluorescent *in situ* hybridization (FISH), which uses fluorescently labeled nucleic acids to visualize complementary sequences by hybridization in both fixed biological structures and living cells (Landstrom and Tefferi, 2006). RNA FISH aims to localize mRNA to its transcription site in a cellular compartment. Quantitative fluorescence *in situ* hybridization (Q-FISH) in combination with flow cytometry, called Flow-FISH, has also been applied to the study of telomere lengths in leukemia cell lines (Levsky and Singer, 2003) using conditions optimized for routine and fast analysis. Work by Levsky and co-workers (Levsky and Shenoy et al. 2002) employing advanced computational fluorescence microscopy and multiplex oligomer DNA probes has demonstrated the feasibility of generating a simultaneous FISH profile for eleven genes in the nuclei of in vitro cultured cells. Furthermore, by using time-lapse video microscopy it was possible to visualize an inducible array of transcription sites, mRNA synthesis and protein products in living cells (Janicki and Tsukamoto et al. 2004).

Several new methods of protein quantitation via Inductively Coupled Plasma Mass Spectrometry (ICP-MS) linked immunoassays (Baranov and Quinn et al. 2002b; Ornatsky and Baranov et al. 2006; Quinn and Baranov et al. 2002) have been developed by our research group and others (Zhang and Zhang et al. 2002), establishing the applicability of ICP-MS-based techniques to the analysis of biological samples. The ICP-MS technique can be applied directly to the sensitive multiplex analysis of bio-molecules with a naturally occurring metal component, while ICP-MS-linked immunoassays provide a means to determine the concentrations of bio-molecules that do not naturally contain a metal (Unwin and Evans et al. 2006). ICP-MS as an analytical detector has a number of unique and relevant properties. The most important advantage is the fact that a large number of heavy metals and their isotopes generate distinct signals that can be detected simultaneously. Thus, many (>50) elemental tags can be created and directed to bio-molecular targets present in a pathological condition (leukemia cell markers, for example); upon detection in the ICP-MS, the obtained intensity ratio vs. tag mass fingerprint could serve as a signature of the disease-state. Second, these signals can be measured over a wide dynamic range, as the abundance sensitivity of ICP-MS, a measure of the overlap of signals of neighboring isotopes, is large ($>10^6$ for the quadrupole analyzer), and this ensures independence of the detection channels. The third key property is that MS is very sensitive; recently we demonstrated that ICP-MS linked immunoassays can be at least as sensitive as radioimmunoassay (Baranov and Quinn et al. 2002a). Finally, an ICP-MS detector offers absolute quantitation that is largely independent of the analyte molecular form or sample matrix, thus simplifying analysis of biological samples (Tanner and Li et al. 2004).

In order to conduct the present gene expression analysis, we have sought out commercially available, metal-tagged reagents currently in use for other applications. Fortunately, there are a variety of metal containing immunoreagents, including gold-tagged antibodies, which are routinely used in electron microscopy. Small gold clusters (1.4 nm diameter Nanogold labeled antibodies) and colloidal gold particles can be successfully analyzed by ICP-MS. In addition, the four lanthanide (Eu(III), Tb(III), Dy(III) and Sm(III)) tagged affinity reagents (Huhtinen and Kivela et al. 2005) produced for fluorescence-based ELISA (Perkin Elmer Life Science, 2005) have been demonstrated to be good candidates for ICP-MS based immunophenotyping (Ornatsky and Baranov et al. 2006) and were used in this study. The present results demonstrate that the ICP-MS approach may be able to provide researchers and clinicians with substantially improved analytical and prognostic capabilities in the identification of gene expression profiles, similar to those seen for protein quantitation and identification.Thus, the development of a highly sensitive, quantitative and multiplex system for gene and protein expression analysis in single cells remains an important goal for molecular research and diagnosis. ICP-MS-based analysis, combined with purpose-specific element tagged affinity reagents, has the potential to achieve this goal.

Experimental Section

Materials and Reagents.

Phosphate Buffered Saline with calcium and magnesium (PBS; 150 mM NaCl, 1.2 mM Ca^{2+}; 0.8 mM Mg^{2+}; 2 mM sodium phosphate, pH 7.4); concentrated 34% HCl (Seastar Chemicals Inc.); Proteinase K, prehybridization and hybridization buffers, 20xSSC (sodium chloride-sodium citrate, pH 7.0 buffer, Ambion); *in situ* hybridization solution (DAKO); PermiFlow solution (InVirion Inc.); 37% formaldehyde and para-formaldehyde (Sigma); Triton X-100 (Sigma); 1 ppb Ir (Iridium) in 1%HCl v/v. All solutions were prepared in distilled water (Millipore) and RNase-free water (Sigma).

Affinity Reagents

Anti-CD33, monoclonal (Immunotech Inc.) used at 1:50 dilution; anti-CD34, monoclonal (BD Biosciences) used at 1:6 dilution; anti-CD38, monoclonal (BD Biosciences) used at 1:40 dilution; anti-BCR rabbit polyclonal antibodies which detect endogenous levels of total BCR and 210 kDa BCR/Abl in K562 cells (Cell Signaling Technologies Inc) used at 1:50; Isotype mouse IgG1, and rabbit IgG (BD PharMingen) were used for negative controls. Gold labeled anti-mouse-Au (Nanogold) was from NanoProbes Inc. Lanthanide-labeled (Eu) affinity reagents were purchased from PerkinElmer Life Sciences (Turku, Finland): anti-rabbit-Eu (DELFIA), was used at 1:200 dilution, and anti-mouse-Eu antibody (DELFIA) was used at 1:100 dilution. StrAv-PerCp (BD Biosciences) and StrAv-Tb (DELFIA). All antibody dilutions were prepared in PBS/10%FBS. Biotinylated oligonucleotide hybridization probes were ordered from Invitrogen Inc.

Cell lines

Human monocyte cell line MBA-1 was derived from Mo7e by retroviral induction of the p210 BCR/Abl cDNA (Sirard and Laneuville et al. 1994). Expression of the BCR/Abl oncogenic kinase confers growth factor independence. MBA-1 expresses moderate levels of CD33 antigen (10,000 copies per cell) and serves as a model system for studying human megakaryocytic leukemia and drug inhibition of the BCR/Abl kinase. KG-1a, acute myelogenous leukemia cell line with high CD34 antigen expression (100,000 copies per cell) and K562, a chronic myeloid leukemia cell line, were obtained from ATCC. Cells were propagated 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 and viability was verified with Trypan Blue (90% viable).

Apparatus. Inductively Coupled Plasma Mass Spectrometry

Experimental measurements for volume analysis were made on a commercial ICP-MS instrument ELAN DRCPlus™ (PerkinElmer SCIEX) described elsewhere (Bandura and Baranov et al. 2001; Bandura and Baranov et al. 2002; Tanner and Li et al. 2004) and operated under normal plasma conditions. The sample uptake rate was adjusted depending on the particular experiment and sample size, typically 100 μl/min. A MicroFlow PFA-ST concentric nebulizer (Elemental Scientific, Inc) was used in all instances. Experiments were performed using an autosampler (Perkin Elmer AS 91) modified for operation with Eppendorf 1.5 ml tubes. Sample size varied from 150 to 300 μl. Standards were prepared from 1000 μg/mL PE Pure single-element standard solutions (PerkinElmer, Shelton, CT) by sequential dilution with high-purity deionized water (DIW) produced using a Elix/Gradient (Millipore) water purification system. In Figures 1–4, 7, the analyte detector signal is normalized to the detector signal of the internal standard 1ppb of Ir added during sample preparation (labeled as normalized response).

Results and Discussion

28S rRNA *in situ* hybridization conditions for suspension cells detected by flow cytometry and volume ICP-MS analysis

In situ hybridization conditions for suspension cells were established using an oligonucleotide DNA probe complementary to the abundant ribosomal 28S RNA species and fluorescent flow cytometry. Several conditions of cell fixation and permeabilization were tested (Figure 1A). Maximum fluorescence intensity was obtained under condition 4 (Figure 1A) using the FACSCalibur (BD

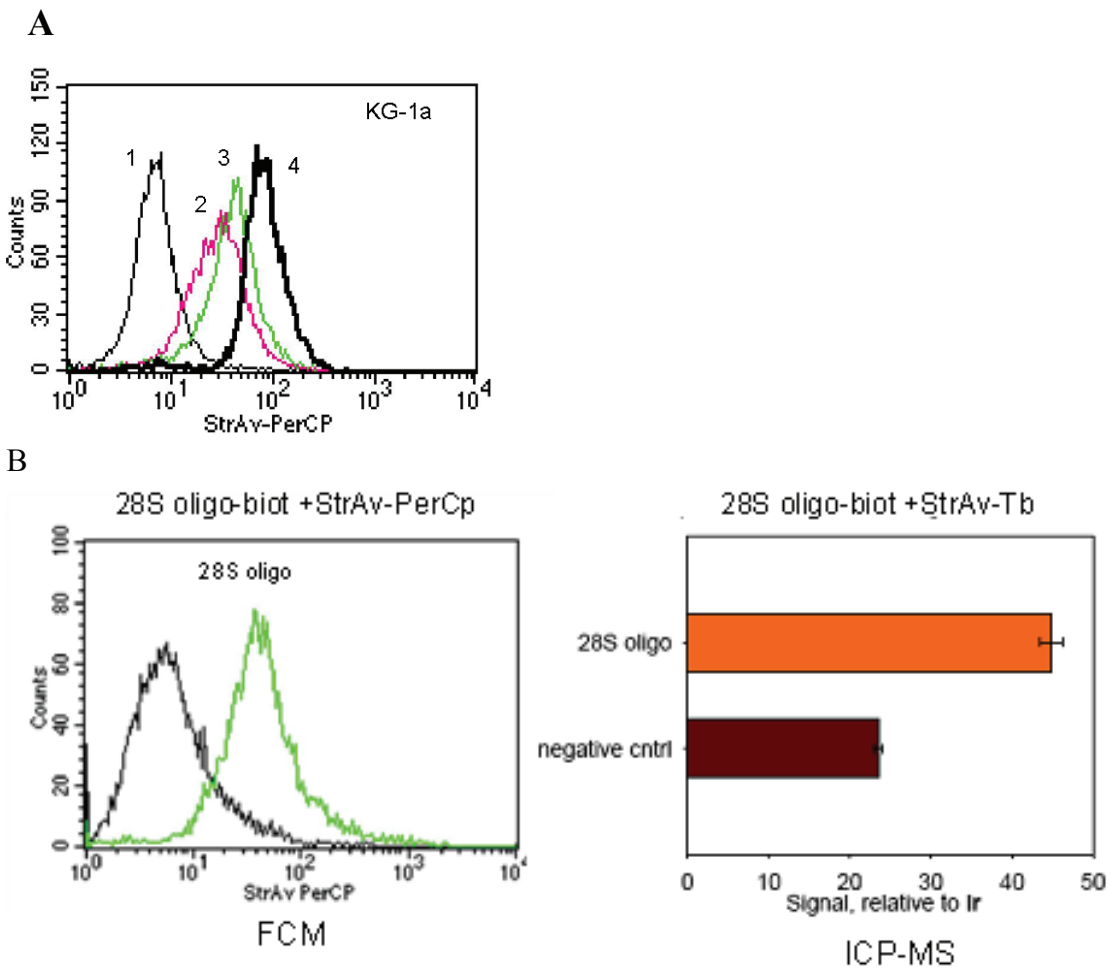

Figure 1.(A) FACS analysis of products obtained using different *in situ* hybridization conditions using biotinylated antisense oligos: (1) non-sense biotinylated oligo, (2) 4% para-formaldehyde 15 min, followed by Proteinase K (5 U/ml) for 15 min at RT and 28S rRNA oligo; (3) 4% para-formaldehyde 15 min and Proteinase K (5 U/ml) for 15 min at 37°C and 28S rRNA oligo; (4) 4% para-formaldehyde 15 min, followed by 0.3% Triton-X100, followed by Proteinase K (5 U/ml) for 15 min at 37°C and 28S rRNA oligo. Conditions denoted by (4) were chosen for further experiments. (B) Comparison of 28S rRNA *in situ* hybridization analyzed by flow cytometry (FCM) and ICP-MS. All samples were prepared in triplicates. Error bars represent the SD of triplicates and reflect variations in sample preparation.

Biosciences) flow cytometer. Cultured KG-1a were collected by low speed centrifugation (500xg, 10 min), washed once with PBS, and fixed in freshly prepared 4% para-formaldehyde/PBS, followed by Proteinase K treatment (5 U/ml) with or without 0.3% Triton-X100. A total of $1x10^6$ cells were treated with prehybridization solution for 30 min at 37°C, then incubated in hybridization solution with 500 ng/ml biotinylated 28S rRNA probe (5'-biotin-ATCCAACGCTTGGTGAATTC-3' Human 28S ribosomal RNA GI:337381) or non-sense biotinylated probe (B/A; negative control) for 2 hours at RT. Following washing and blocking in PBS/1%BSA, StrAv-PerCp was added for 30 min. These fixation/permeabilization conditions and *in situ* hybridization parameters for KG-1a cells were subsequently used for ICP-MS gene expression analysis. For ICP-MS volume analysis, MBA-1 cells after hybridization with the 28S rRNA biotinylated probe were reacted with StrAv-Tb, washed and dissolved in HCl conc. with 1ppb Ir added as internal standard. Thus a batch of KG-1a cells was hybridized with 28S biotinylated oligos (green line histogram Fig.1B FCM) and orange bar Fig.1B ICP-MS) or non-sense biotinylated oligos (black line histogram Fig.1B FCM) and brown bar (Fig.1B ICP-MS). After extensive washing with 4xSSC, 1xSSC, and 0.2xSSC, cells were split into aliquots: cells for flow cytometry were reacted with StrAv-PerCp, while cells for ICP-MS were reacted with StrAv-Tb. As can be seen from Figure 1B, there is a clear hybridization signal for 28S rRNA detected by flow cytometry (FCM) and mass spectrometry (ICP-MS) simultaneously. Notice that the two

methods are very different and can be compared only approximately. ICP-MS method is based on analysis of sample volume yielding a measurement directly proportional to an analyte concentration in the whole sample. SD of this type of measurement is very small and close to the counting statistics limit. Flow cytometry method is based on measurement of an analyte concentration per single cell and SD of the method is characteristic of deviation of an analyte concentration in the cell population.

Leukemia-specific and ubiquitous transcript identification in myeloid cells by ICP-MS

Specificity of BCR/Abl mRNA detection. K562 cells which are known to express the b3a2 variant BCR-Abl p210 mRNA and KG-1a that do not (Guo and Lin et al. 2001) were selected as a model system. The following reagents were used: 5'-biotinylated b3a2 fusion probe (biot-5'-CTCAAGTTTTCGGGAAGTCG-3' GI:4033554), and the 5'-biotinylated 28S rRNA probe as positive control. Cells were fixed in 4% para-formaldehyde, permeabilized with 0.3% Triton-X100 and treated with Proteinase K as described above. Cells were then incubated in hybridization solutions (Ambion) containing 700 ng/ml of biotinylated BCR/Abl probe, 28S rRNA, non-sense probe or no probe at all for 2h. Following washing and blocking in PBS/0.5%BSA, StrAv-Tb was added. The first round of analysis was carried out by volume ICP-MS analysis where labeled cells were dissolved in HCl/Ir and the entire cell population ($0.3x10^6$cells/vial for KG1a and $3x10^6$ cells/vial for K562) was subjected to elemental analysis by conventional ICP-MS. Results are presented in Figure 2. The graph in Figure 2A demonstrates that while the level of 28S rRNA in KG-1a cells is very high, the signal for the BCR/Abl probe is low and is at the levels of non-sense (B/A) and negative control (ctrl) normalized responses. On the other hand, K562 cells (Figure 2B) show a high expression of BCR/Abl mRNA, although it is approximately 14-fold lower than 28S rRNA expression (background, negative control values subtracted). Thus, metal-tagged detection of BCR/Abl gene expression in K562 cells by ICP-MS is reliable and specific.

Identification of ubiquitous gene transcripts by ICP-MS. Expression analysis of constitutive (actin) and proliferation related (D-cyclin) mRNAs was performed on actively growing K562 cells similarly to the above described experiments. 5'-biotinylated 21-mer oligos for conserved regions of human D-cyclin (5'-biot-AGGAAGCGGTCCAGG-TAGTTC-3' GI:77628152) and human cytoskeletal actin (5'-biot-AGCACTGTGTTGGCGTACAG-3' GI:12654910) were used in addition to BCR/Abl biotinylated probe. A mouse cytoskeletal actin biotinylated probe (5' biot-AAGGAAGGCTG-GAAAAGAGC-3' GI:5016088) was used as a negative control in this set of experiments. Results are shown in Figure 3. Significantly higher levels are detected for human specific actin (H) transcripts compared to mouse actin (M). D-cylclin (D) transcript is the most abundant mRNA species in these actively growing K562 cells while the BCR/Abl (B) fusion transcript is present at slightly lower levels.

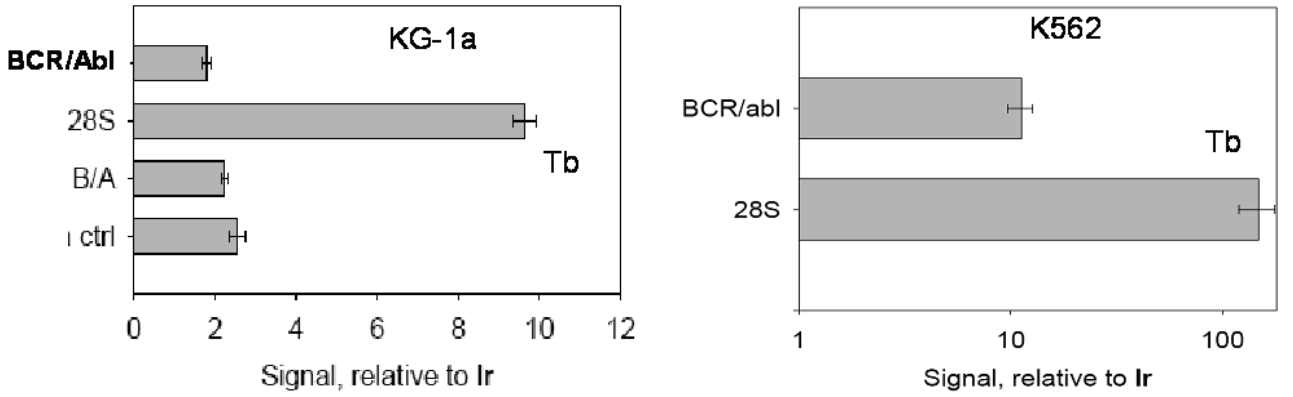

Figure 2. BCR/Abl gene expression analysis by ICP-MS. Suspension cultured cells were *in situ* hybridized after fixation and permeabilization with a biotinylated probe for BCR/Abl fusion gene. Biotin was identified by streptavidin (StrAv) labeled with lanthanide (Tb). Cell pellets were dissolved in HCL and analyzed by conventional ICP-MS. Experimental results for KG-1a cells (A) and K562 cells (B, background and non-sense probe response values subtracted) hybridized with BCR/Abl antisense, 28S rRNA (positive control) and non-sense oligo probes (B/A) and no probe (background control, ctrl). Error bars represent the SD of triplicates and reflect variations in sample preparation.

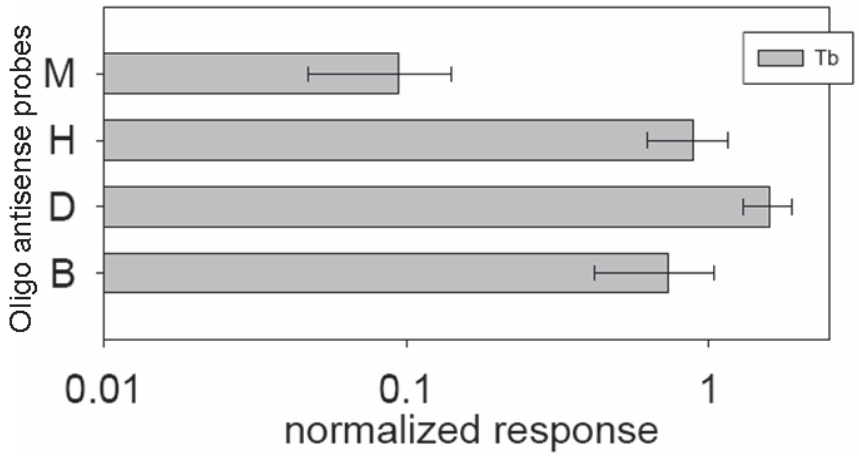

Figure 3. Identification of abundant mRNA species in K562 cells by ICP-MS *in situ* hybridization. (M)—Mouse cytoskeletal actin probe; (H)—human cytoskeletal actin probe; (D)—human D-cyclin probe, and (B)—BCR/Abl probe. Values are given as normalized response (relative to Ir internal standard) and background (no probe) subtracted. Error bars represent the SD of triplicates and reflect variations in sample preparation.

Simultaneous gene and protein detection in K562 leukemia cells

To test simultaneous gene and protein identification by volume ICP-MS, we used the p210 BCR/Abl expressing cell line K562. The oncogenic kinase BCR/Abl and total BCR were identified by primary antibodies raised in rabbit, anti-BCR, while the cell surface antigen CD38 was detected via the mouse monoclonal anti-CD38 antibodies. The b3a2 fusion gene transcript was hybridized with the biotinylated BCR/Abl olionucleotide probe as mentioned above. In this series of experiments, $3x10^6$ K562 cells per treatment were fixed and permeabilized in PermiFlow 1^x solution for 40 min at RT, washed in 10%FBS/PBS and incubated with a mixture of primary anti-BCR and anti-CD38 antibodies (30 min at RT). The immunoreacted cells were then washed and incubated for 2 hrs with 700 ng/ml of biotinylated BCR/Abl oligo probe in DAKO *In situ* Hybridization solution. Successive washes in 4xSSC, 2xSSC, 0.2xSSC were followed by a brief incubation in blocking solution (10%FBS/PBS). Finally, the cells were reacted with a mixture of secondary affinity reagents: anti-rabbit-Eu, anti-mouse-Au antibodies, and StrAv-Tb. Thus, b3a2 fusion gene was labeled by Tb; p210 BCR/Abl protein was labeled by Eu, and the surface marker CD38–by Au. Finally, excess metal-chelated secondary antibody was washed out from the cells by several incubations in PBS and low speed centrifugation. The cell pellets were dissolved in HCl prior to ICP-MS analysis. Results of the volume ICP-MS analysis are given in Fig.4. Since control tubes treated with isotype IgG and the same amount of secondary metal-tagged antibodies show lower detection signals, we conclude that proteins in the test samples were specifically identified. As evident from the graphs, distinct signals from the BCR/Abl transcript together with the surface and intracellular protein markers of the K562 cells could be registered simultaneously by volume ICP-MS. Somewhat lower signals obtained in this series of experiments may be explained in part by the "stickiness" of Au, thus increasing background noise, and by the use of secondary reagents for target detection. Directly metal conjugated specific oligos and primary antibodies will substantially increase the signal-to-noise ratio as well as obviate the problem of having to use a limited range of secondary antibodies. Currently, our group is developing novel tagging reagents that carry a predefined number of lanthanide metals and can be covalently linked to affinity reagents. The directly labeled reagents will permit us to perform truly multiplex experiments with up to 50 antibodies and oligo probes in one tube.

As the prototype flow-ICP-MS instrument becomes available, the same experiments will be set up and single-cell analysis of myeloid leukemia gene and protein expression will be performed.

Conclusions

The present work demonstrates the feasibility of using metal-tagged *in situ* hybridization for mRNA

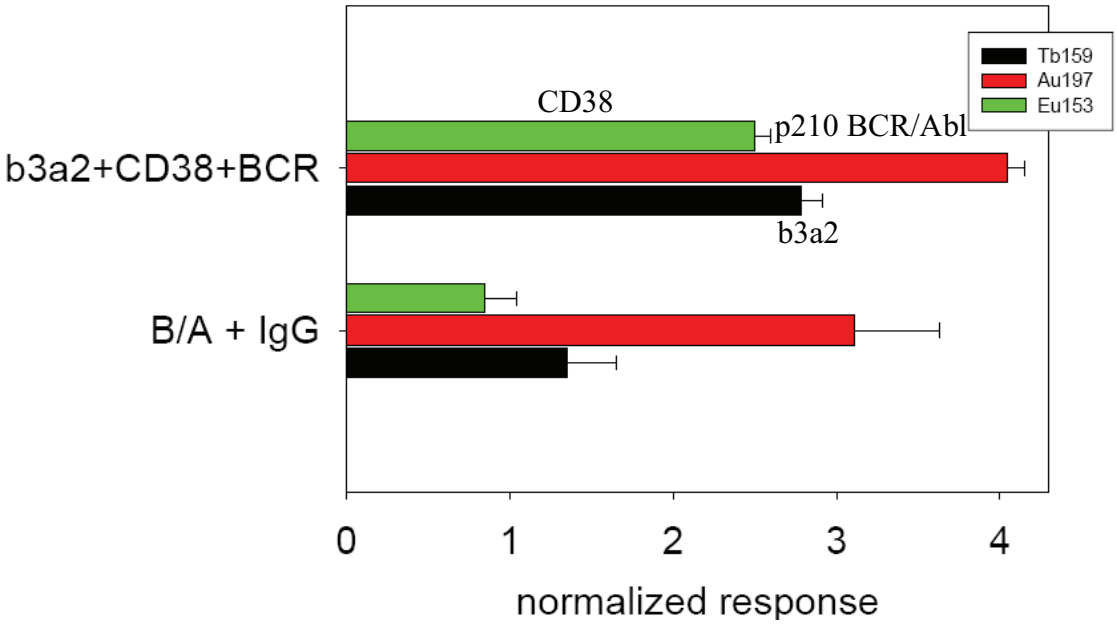

Figure 4. Simultaneous gene transcript and protein identification in chronic myeloid leukemia cells by ICP-MS. K562 cells were fixed/permeabilized and incubated with primary anti-CD38 and anti-BCR antibodies, followed by *in situ* hybridization with a biotinylated probe b3a2 specific for the BCR/Abl gene. Secondary antibodies tagged with metals (anti-rabbit-Eu and anti-mouse-Au) were used to identify proteins (CD38 = Au and p210 BCR/Abl = Eu) as well as gene transcripts (BCR gene = Tb) using StrAv-Tb in the same cells. Sample labeled as B/A+IgG corresponds to control cells incubated with isotype mouse, rabbit immunoglobulins and non-sense biotinylated oligo B/A. Error bars represent the SD of triplicates and reflect variations in sample preparation.

detection by ICP-MS. We show that by employing commercially available metal-tagged antibodies and metal-tagged streptavidin, and a commercial ICP-MS instrument (ELAN DRCPlus^{TM}), moderately and highly abundant mRNA transcripts could be identified in model leukemia cell lines. Multiplexing will be achieved by labeling oligonucleotide probes with different metal-containing tags which are currently being developed in our group. These metal-tags will also be attached to antibodies and following the protocols outlined above, simultaneous multiplex gene and protein identification should be possible, thus increasing the read out of a diseased cell biomarker profile necessary for classification, diagnosis and prognosis of leukemia patients. Furthermore, sensitivity of mRNA detection can be improved by using three to five oligo DNA probes per transcript with each probe labeled with the same element. Our group is developing a novel ICP-MS-based flow cytometer which will allow multiplex gene expression profiling of each leukemia cell in a patient's blood sample. Hybridization of oligos on fluorochrome coded beads (Han and Gao et al. 2001; Kohara, 2003) and nanoparticles (Rosi and Mirkin, 2005) has become an alternative approach to slide arrays for gene expression analysis. Analogous to these beads, we are developing polystyrene microspheres imbibed with different metals or combinations of metals. Figure. 5 displays the proposed work-flow. According to this approach, leukemia cells should be isolated from bone marrow or peripheral blood and total RNA extracted by standard molecular biology techniques; *in situ* hybridization will be performed with specific oligonucleotide probes attached to metal-coded microsperes. Since the number of elements and their stable isotopes that can be used is around 50 and each type of microsphere may be imbibed by a unique combination of metals, the number of different specific probes linked to uniquely coded beads may be very large (10^5–10^6 different coded beads are feasible). After stringent washing of hybridized probe-mRNA duplexes, oligo(dT)n-metal will be added to indicate the presence of mRNAs on the beads. Finally, the ICP-MS-based flow cytometer should interrogate each bead one at a time for the elemental signals of the specific probes and mRNA hybridization. Sensitivity of the elemental analyzer should not be

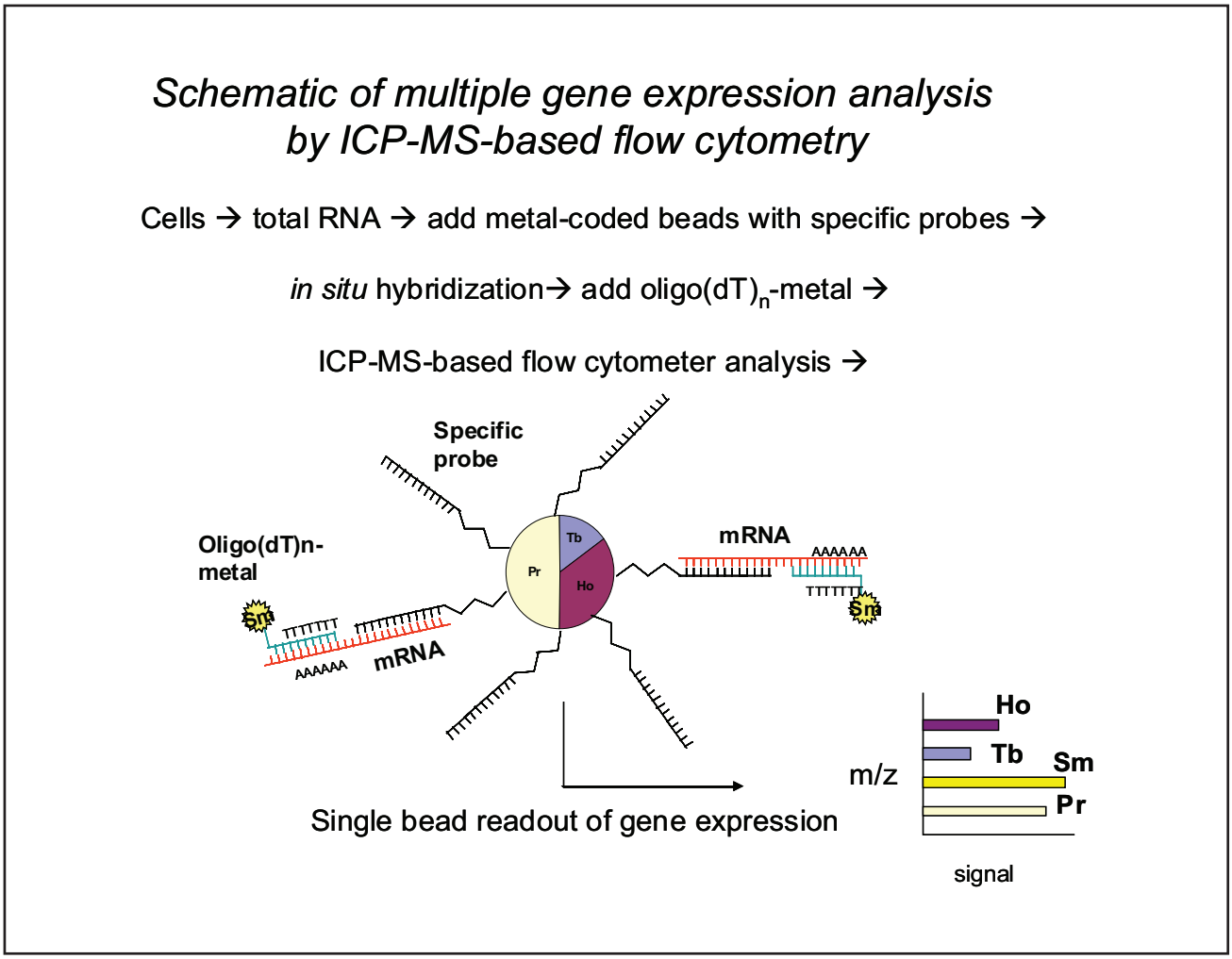

Figure 5. Schematic of multiple gene expression analysis by ICP-MS-based flow cytometry. Description is given in the text.

a limiting problem. In this paper we have demonstrated that 4–6 atoms of lanthanide per tag were sufficient for detection whereas the element code of beads could be in the order of several thousand atoms per bead. Thus, we expect that the ICP-MS-based flow cytometry will permit sensitive, quantitative and highly multiplexed gene analysis of acellular biological samples such as body fluids and isolated nucleic acid preparations.

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