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A platinum-based covalent viability reagent for single cell mass cytometry

Harris Fienberg[1,2,4,*], **Erin F. Simonds**[1,2,*], **Wendy J. Fantl**[1,2], **Garry P Nolan**[1,2,5,#,‡], and **Bernd Bodenmiller**[1,2,3,6,#]

[1]Baxter Laboratory for Stem Cell Biology, Stanford University School of Medicine, Stanford, CA

[2]Department of Microbiology & Immunology, Stanford University School of Medicine, Stanford, CA

Abstract

In fluorescence-based flow cytometry, cellular viability is determined with membrane-impermeable fluorescent reagents that specifically enter and label plasma membrane-compromised non-viable cells. A recent technological advance in flow cytometry uses antibodies conjugated to elemental metal isotopes, rather than to fluorophores, to allow signal detection by atomic mass spectrometry. Unhampered by the limitations of overlapping emission fluorescence, mass cytometry increases the number of parameters that can be measured in single cells. However, mass cytometry is unable to take advantage of current fluorescent viability dyes. An alternative methodology was therefore developed here in which the platinum-containing chemotherapy drug cisplatin was used to label cells for mass cytometry determinations of live/dead ratios. In a one-minute incubation step, cisplatin preferentially labeled non-viable cells, from both adherent and suspension cultures, resulting in a platinum signal quantifiable by mass cytometry. This protocol was compatible with established sample processing steps for cytometry. Furthermore, the live/dead ratios were comparable between mass and fluorescence based cytometry. Importantly, although cisplatin is a known DNA-damaging agent, a one-minute “pulse” of cisplatin did not induce observable DNA damage or apoptotic responses even within 6 hours post-exposure. Cisplatin can therefore be used as a viability reagent for a wide range of mass cytometry protocols.

Keywords

Mass cytometry; cisplatin; viability reagent

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[‡]Correspondence should be addressed to Garry P. Nolan. gnolan@stanford.edu, 269 Campus Dr., CCSR 3205, 94305 Stanford, CA. Phone (650) 725-7002, fax: (650) 723-2383.
[*,#]These authors contributed equally to this work.
[3]Current address: Institute of Molecular Life Sciences, University of Zurich, Switzerland

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Introduction

Determination of cellular viability is a critical metric in flow cytometry experiments as it enables the identification and removal of dead cells that could confound functional studies of intracellular signaling, apoptosis, DNA damage, cancer, and drug responses. Fluorescence-based cytometry utilizes a variety of approaches to determine cellular viability status, the most popular of which are “viability dyes”. These molecules, including 7-Aminoactinomycin D (7-AAD)(1) and propidium iodide(2) cannot pass through the intact membrane of a live cell, but efficiently enter the cytoplasm and nuclei of dead cells where they intercalate non-covalently into DNA generating a measureable fluorescent signal. Such compounds are effective for discriminating live and dead cells, but are washed out in subsequent cell permeabilization steps needed for well-established intracellular staining protocols(3).

Covalent protein-reactive fluorescent dyes (*e.g.*, Invitrogen Fixable LIVE/DEAD (Aqua stain))(4) withstand the formaldehyde crosslinking and permeabilization steps and can be used when many sample processing steps are performed.

Other approaches allow the determination of more specific aspects of apoptotic cell death. Propidium iodide can be used to determine DNA fragmentation occurring during apoptosis(5). Here cells are fixed, permeabilized and treated with RNAse and subsequently stained with propidium iodide. As small DNA fragments generated during apoptosis leak out of the permeabilized cells, the apoptotic cells will yield a lower signal compared to viable cells in the G1, S or G2 phases of the cell cycle. Alternatively, the TdT dUTP nick end labelling (TUNEL) assay can be used to determine DNA fragmentation(6). Here a terminal deoxynucleotidyl transferase labels DNA nicks with fluorescently labeled 2´-deoxyuridine 5´-triphosphate (dUTP), which can be detected by flow cytometry(6).

Changes in the mitochondrial transmembrane potential, an early hallmark of apoptosis(7), can be determined by measuring differences in fluorescence of the monomeric and aggregate forms of 5,5’,6,6’-tetrachloro-1,1’,3,3’ tetraethylbenzimidazolylcarbocyanine iodide (JC-1), which exists as both a monomer in the cytosol (green) and as aggregates in the mitochondria (red) in non-apoptotic cells, but remains monomeric in the cytosol of apoptotic cells.

Another major class of viability indicators consists of fluorochrome-conjugated antibodies or affinity binders against apopototic specific markers, e.g. against cleaved caspase 3 and cleaved poly-(ADP)-ribose polymerase (PARP) as well as fluorochrome-conjugated Annexin V protein(8–12).

Elemental mass spectrometry-based cytometry (mass cytometry(13–18)) is a recently developed technology platform that characterizes single cells with binding reagents, such as antibodies, aptamers, chemical linkers, or other affinity reagents, in which fluorochromes have been replaced with elemental metal isotopes(17,18). Mass cytometry measures the quantity and mass of stable metal isotopes attached to cells via affinity binders, each labeled with polymeric, metal-loaded chelators. The detection modality in mass cytometry is an inductively-coupled plasma mass spectrometer (ICP-MS), which in the current generation of instruments allows for the analysis of spectra encompassing up to 100 isotope channels at an acquisition rate of 1000 cells/second(18). The high resolution of the time-of-flight (TOF) mass analyzer used in the mass cytometer combined with the intrinsically discrete nature of isotopic masses allows discrimination of isotopes separated by only one atomic mass unit with negligible spectral overlap and dramatically increases the number of parameters that can be measured simultaneously per single cell.

To date, three approaches for determining cell viability by mass cytometry have been described. Ornatsky et al. demonstrated two custom-synthesized (now commercially available) rhodium- and iridium-containing metallointercalators that were membrane-impermeable[14]. Similar to propidium iodide, these reagents do not form covalent bonds with cellular components, and are thus subject to washout during sample processing. As such, these reagents can only be used as viability dyes when staining is performed shortly before data acquisition. Subsequently, Bendall et al.[17] used an amine-reactive chelator, 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid mono (N-hydroxysuccinimide ester) (DOTA-NHS-ester) loaded with Rh(III) to label dead cells. This reagent behaves analogously to the amine-reactive fluorescent reagents, but requires same-day preparation in order to produce reproducible results. In a third approach, Majonis et al. described the preferential labeling of dead cells by EDTA polymers loaded with palladium (Pd) or platinum (Pt) ions, conjugated to either antibodies or fluorescein[19]. The authors postulated that some Pd or Pt ions exchange the EDTA chelator in favor of stronger chelating reactions with ligands in the cell, but they did not test whether this reagent is stable enough to withstand a high number of stringent sample processing steps. Moreover, these newly developed EDTA-based reagents are not yet commercially available.

Cisplatin [$Pt(NH_3)_2Cl$] is a readily available, platinum-based chemotherapeutic agent, which is used to treat a variety of cancers(20). After accessing the cell interior, mainly by passive diffusion, cisplatin's cytotoxicity stems from its ability to form 1,2- and 1,3-intrastrand crosslinks between purine bases of DNA, which stalls replication and transcription, resulting in a DNA damage response, cell cycle arrest and ultimately apoptosis(20,21). However, in this study, these molecular properties were not utilized to measure cell viability. Instead, cisplatin's rapid entrance into a cell with a compromised plasma membrane, followed by its rapid reactivity towards protein nucleophiles such as R-SH or R-S-CH_3(20,21), with which it can form covalent Pt-S bonds were the features exploited by the viability measurements (Figure 1A). In a slower, but energetically more favorable process, cisplatin can also become activated by aquation(22), forming $[Pt(NH_3)_2Cl(OH_2)]^+$ and $[Pt(NH_3)_2(OH_2)_2]^{2+}$, enabling it to form a covalent bond with N7 position of purine bases in DNA (Figure 1A).

We present the use of cisplatin to quantitatively discriminate live from dead cells in mass cytometry assays. Furthermore, the data generated with cisplatin were comparable to those generated with fluorescent viability dyes.

Materials and Methods

Cell culture

The cancer cell lines Jurkat (T acute lymphoblastic leukemia suspension cells) and OVCAR-3 (ovarian carcinoma adherent cells) were cultured in RPMI-1640 (GIBCO, Invitrogen, Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/ml penicillin. The suspension cell lines KG-1 (acute myeloid leukemia) and HL-60 (acute myeloid leukemia cells) were cultured in IMDM (GIBCO, Invitrogen, Carlsbad, CA) with 20% heat-inactivated FBS and 100 U/ml penicillin. The adherent cell line HeLa (cervical carcinoma) was cultured in DMEM (GIBCO, Invitrogen, Carlsbad, CA) with 10% heat-inactivated FBS and 100 U/ml penicillin. All cell lines were maintained at 37°C in a humidified 5.0% CO_2 environment. Peripheral blood from healthy donors was purchased from the Stanford Blood Bank according to an IRB-approved protocol. Peripheral blood mononuclear cells (PBMCs) were separated over Ficoll-Paque Plus (Amersham Biosciences) using Accuspin tubes (Sigma-Aldrich, St. Louis, MO) to remove erythrocytes, platelets, and granulocytes. PBMCs were then suspended in FCS with 10% DMSO and frozen at the temperature of liquid nitrogen.

Generation of non-viable control cells

To generate samples with known amounts of non-viable cells, an aliquot of cultured cells was heat-killed at 55°C for 1 hour, cooled to 37°C and then spiked into each culture of viable cells at known percentages based on Trypan blue exclusion staining(23). Samples were then prepared for fluorescence and mass cytometry and the live/dead ratio of cells determined by each technology.

To assess the dynamic range and reproducibility of the viability assay, heat killed HL-60 cells were serially diluted in 2-fold steps and spiked into a cultures with equal amounts of viable HL-60 cells, to generate samples with a final concentration of 40%, 20%, 10%, 5%, 2.5% and 1.25% non-viable cells. Then live/dead cell ratios were determined by Trypan blue exclusion for samples with greater than 5% dead cells. Below this percentage, determining the abundance of dead cells by Trypan blue exclusion is unreliable. Therefore, at low percentages, the number of dead cells was extrapolated based on the serial dilution. Each cell aliquot was divided into triplicates (2×10^6 cells/ml), exposed to the cisplatin viability reagent and processed for mass cytometric measurement as described below.

Cisplatin exposure

Cisplatin (WR International, Radnor, PA, Cat# 89150-634) was stored at −80°C as a stock solution of 100 mM in DMSO (Hybrimax, Sigma Aldrich, St Louis, MO). Working solutions (10 mM) were prepared fresh on the day of each experiment by diluting the stock solution into PBS at 4°C. Cells in suspension were centrifuged at 300×g for 5 minutes and resuspended in 1ml serum-free RPMI at 2×10^6 cells/ml. The cisplatin working solution was added to cells at a final concentration of 25 μM for 1 min at room temperature, The reaction was quenched with 3ml of RPMI/10% FBS. Samples were then centrifuged at 300×g for 5 min and cell pellets were resuspended in 1 ml RPMI/10% FBS and processed for cytometry.

Pervanadate stimulation of PBMCs

Frozen PBMCs were thawed at 37°C and resuspended in RPMI-1640/10% FBS/ 2mM EDTA. A fraction of the PBMCs (30%) was heat-killed separately and spiked back in, as described above. Cells were treated with cisplatin according to the standard protocol described above at both 10^6 cells/ml. Samples were then treated with activated sodium orthovanadate at a final concentration of 125 μM for 15 min at 37°C. Cells were fixed with 1.6% (final concentration) PFA for 10 min at room temperature.

DNA damage response determination

KG-1 cells (2×10^6 cells/ml) were exposed to 25 μM cisplatin for 1 min as described above. After quenching, cells were centrifuged at 300×g for 5 min, resuspended at 2×10^6 cells/mL in RPMI/10% FBS and incubated at 37°C. In order to evaluate whether cisplatin exposure mediated DNA damage, a 1 mL aliquot of cells was removed, fixed in PFA and washed in PBS at times 30 min, 60 min, 120 min, 240 min and 360 min post cisplatin treatment and quenching. Samples were then permeabilized and incubated with antibodies as described.

Antibody staining

After fixation, cells were permeabilized with methanol for 10 min at 4°C, washed twice in cell staining media (CSM; PBS with 0.5% bovine serum albumin and 0.02% sodium azide), and then incubated for 30 min at room temperature simultaneously with relevant antibodies. KG-1 cells were incubated with antibodies against pH2AX and cleaved poly-ADP ribose polymerase (cPARP) to mark cells that had undergone DNA damage and/or apoptosis. PBMCs were incubated with antibodies against surface markers to delineate immune cell subtypes and pSLP-76, an intracellular signaling molecule and substrate for ZAP-70(24).

For PBMCs treated with pervanadate the antibodies shown in table 1 were used. For KG-1 cells undergoing DNA damage determination the antibodies shown in table 2 were used. After antibody incubation, cells were washed once in cell staining media (CSM), stained with 1 mL of 1:5000 $^{191/193}$Iridium (Ir) DNA intercalator (www.dvssciences.com; DVS Sciences, Richmond Hill, Ontario, Canada), diluted in PBS with 1.6% PFA and incubated for 20 min at room temperature or at 4°C overnight. Cells were then washed twice with CSM and finally with water for mass cytometric analysis. In order to accurately assess the durability of cisplatin staining in the absence of antibodies, a mock antibody staining procedure was performed with a 30 minute incubation step in 100 μL CSM followed by all subsequent sample processing steps as described above.

Mass cytometry measurement

Cells were analyzed on a CyTOF® mass cytometer (www.dvssciences.com; DVS Sciences, Richmond Hill, Ontario, Canada). The settings of the instrument and the initial post-processing parameters have been described previously (17,18). Cells were measured at approximately 500 cells per second, noise reduction was activated and cell extraction parameters were set to: Cell length, range was set from 10 to 65 pushes, and the lower convolution threshold was set to 10.

Data repository and MIFlowCyt compliance

Experiments were performed between July and August 2011. Complete listmode (.FCS) files and gating strategies from these experiments may be viewed or downloaded at http://cytobank.org/nolanlab

Data analysis and visualization

All cell density plots and histograms shown were created in Cytobank (www.cytobank.org, Cytobank, Inc., Menlo Park, CA). After publication all FCS files, including data and time of the experiment, will be made publicly available via Cytobank. All parameters were displayed with an arcsinh transformation, a scale argument of 5, and a display range from −20 to 20,000.

Results

Comparison between fluorescence and mass cytometry protocols

The performance of cisplatin at different concentrations to determine the live/dead cell ratio by mass cytometry was benchmarked against the current standard fluorescent viability stain, Aqua. In the first experiment a suspension of human myeloid KG-1 cells spiked with approximately 30% of heat-killed KG-1 cells (determined by Trypan blue extrusion), was tested with Aqua and cisplatin. The data shown in Figure 1B verified the ability of Aqua to identify the expected 30% dead cell population.

Cells treated for 1 min with cisplatin showed increased platinum staining with increasing cisplatin concentration (10, 50, or 250 μM), but importantly, showed a comparable frequency (26 – 28%) of cisplatin positive (dead) cells at all doses. At higher concentrations, the platinum-low (i.e., “live”) cell population was labeled with detectable amounts of platinum probably due to non-specific labeling (Figure 1C, far right hand panel and discussion). The percentages of cisplatin-positive cells were consistent with the percentages of Aqua positive cells (26%) determined from fluorescence cytometry and suggested that cisplatin and Aqua were preferentially labeling the same population of dead cells (Figure 1B, C). These data validate the use of cisplatin as a viability reagent for use in mass cytometry protocols.

Adaptability of cisplatin as a viability indicator for suspension and adherent cell lines

In order to determine whether the use of cisplatin could be applied to the mass cytometry workflow for other cell types, it was tested for its ability to recognize live/dead cell ratios in two adherent cell lines; HeLa and OVCAR3 and two suspension cell lines; Jurkat, and KG-1. Suspensions of all cell lines were spiked with a known percentage (as determined by Trypan blue extrusion) of heat-killed cells of the same cell type and the live/dead cell frequencies were measured by mass cytometry of cells exposed to cisplatin at three concentrations (Figure 2). Distinct cisplatin-high (dead) and cisplatin-low (live) populations were visible at each cisplatin concentration (Figure 2), with signal-to-noise ratios above 10. The larger, adherent cell lines (OVCAR and HeLa) exhibited higher cisplatin uptake at all concentrations. Indeed, the heat-killed adherent cells acquired so much cisplatin at the highest concentration (250 μM) that the upper limit of the mass cytometer's dynamic range was reached, saturating the signal of the detector and hampering the univocal assignment of viability status at high cisplatin concentrations. These data established an optimal concentration of ~25 μM for viability determinations in cell lines, which was used in all subsequent experiments. These data show that the use of cisplatin for mass cytometry measurements of live/dead ratios will be generally applicable to multiple cell types.

Dynamic range and reproducibility of cisplatin

As with any routine assay, the application of cisplatin to determine live/dead cell ratios needs to be reproducible and to cover a wide dynamic range. In order to test these parameters, triplicate samples were generated in which a two-fold serial dilution of non-viable HL-60 cells was added to a constant number of viable cells, yielding a titration from 40% to an estimated final concentration of 1.25% (0%) dead cells. These live/dead cell mixtures were then analyzed by the cisplatin viability reagent and mass cytometry. The replicates shown in Figure 3 establish that mass cytometry of cisplatin-labeled cells reproducibly determined the percentage of dead cells in all samples with standard deviations ranging from 0.18 (sample with 2.5% dead cells) to 0.95 (sample with 40% dead cells) (Figure 3). The cisplatin assay reliably detected dead cells over the dynamic range of 2.5 – 40% as analyzed in this experiment. The mass cytometry cisplatin assay was reliable at the upper and lower frequency ranges. Importantly, the assay reproducibly detected low endogenous levels of non-viable cells within the sample, i.e. ~2% dead cells when no heat killed cells were spiked in. This attribute of the assay is essential for determining sample quality in any situation, especially in those measuring intracellular signaling responses in cell subsets within a sample.

Cisplatin as a viability indicator for primary samples

Unlike cell lines, primary samples are mixtures of cell subsets with different sizes and morphologies. To test whether cisplatin can identify dead cells within primary samples, primary PBMCs cryopreserved from a healthy donor with spiked in heat- killed cells were subjected to the cisplatin protocol. In addition, since intracellular signaling responses form an integral part of multiparametric cytometry studies and only occur in viable cells, cisplatin viability measurements were made within the context of signaling(17). Therefore to evaluate signaling in PBMCs, phosphorylation of SLP-76 (Y128) (T cells and myeloid cells) and SLP-65 (Y76) (B cells) was determined after a 10 minute exposure to vanadate, a global tyrosine phosphatase inhibitor, shown to increase intracellular kinase activity and phosphotyrosine levels.

Distinct cisplatin-low and cisplatin-high populations were seen in populations of myeloid cells, T cells and B cells (Figure 4A). Furthermore, while cisplatin-low populations of myeloid cells, T cells and B cells showed a strong increase of SLP-76/65 phosphorylation in response to pervanadate, the cisplatin-high populations showed no response (Figure 4A and

Figure 4B). These data show that cells in which detection of platinum is low are proficient in eliciting a signaling response whereas those where platinum detection is high have lost this proficiency. This validates cisplatin as a viability reagent that can be used to computationally gate or remove dead cells that could confound signaling response data.

Measurement of DNA damage and apoptosis

Cisplatin is used clinically as an anti-cancer agent. Its efficacy is based on its ability to mediate a DNA damage response (DDR) and apoptosis. For all experiments in which a quantitative measurement of viability is necessary, the induction of DDR could preclude the use of cisplatin. To determine whether the cisplatin labeling protocol induces DDR and/or apoptosis, a time course experiment (30 minute – 6 hours) was performed for KG-1 cells measuring DDR and apoptosis post a 1 minute pulse of cisplatin. Cells were removed at indicated times, washed and incubated with isotope-tagged antibodies against H2AX (pS139) and cPARP, to measure DDR and apoptosis respectively (Figure 5). The cisplatin-treated cells showed no increase in H2AX phosphorylation or PARP cleavage up to 6 hours following a one-minute exposure to 25 μM cisplatin (Figure 5). As a control, KG-1 cells exposed to 20 μM etoposide continuously for 9 hours showed a 4.5 fold increase in the frequency of cPARP+/pH2AX+ cells. These results demonstrate that a rapid cisplatin exposure for 1 minute does not induce DDR and apoptosis in live cells.

Discussion

The data reported in this study have described a technically straightforward protocol to measure live/dead ratios in single cells by mass cytometry using commercially available cisplatin. The assay developed was reproducible, demonstrated a wide dynamic range, was compatible with many cell types, and allowed for routine processing steps necessary to prepare samples for multi-parameter flow cytometry(17). Critically, the data from fluorescence and mass cytometry were comparable (Figure 1B and Figure 1C). The assay data showed that signaling responsiveness, as determined by exposure of cells to vanadate, was severely diminished in cells that were positive for cisplatin staining. Taken together the data describe a means to gate out dead cells which would confound quantification of signaling response and many other types of data.

In order for the cisplatin methodology to be incorporated routinely into mass cytometry protocols, it was evaluated in adherent and suspension cell lines as well as in primary samples. Although, the concentration-dependent labeling mechanism resulted in different cisplatin intensities based on cell size, the frequency of cisplatin containing cells within samples of different cell lines was equivalent (Figure 2). Furthermore, the concentration of cisplatin chosen from the cell line experiments (25 μM), clearly distinguished live from dead cells within different immunophenotypic cell subsets in a primary PBMC sample (Figure 4). However, given the vast number of biological systems amenable to single cell mass cytometry assays, it would be recommended that cisplatin be titrated by each investigator for their system of interest.

In comparison to Trypan blue exclusion assays, cisplatin labeling returned slightly lower than expected concentrations of dead cells at high live/dead cells ratios and slightly higher than expected concentrations of dead cells at low live/dead cell ratios (Figure 3). Likely, two factors are contributing to this outcome. First, as samples are processed for mass cytometry, multiple centrifugation steps preferentially remove dead cells, which are less dense than live cells. This effect is especially pronounced in samples with high percentages of dead cells. Second, the live cell culture used in our serial dilution contained a small percentage of endogenous dead cells (approximately 1.9% by cisplatin staining), which contributed to a greater than expected number of dead cells throughout the serial dilution. This was not

accounted for in the extrapolation for samples with low live/dead cell ratios. In spite of the small discrepancies between the comparison of Trypan blue and mass cytometry approaches, cisplatin/mass cytometry is the method of choice as it measures live/dead ratios simultaneously with other cell parameters. The lower recovery of cells seen at high dead cell frequencies suggest that it will be important to include other measurements of activated cell death in the assays, particularly events occurring before the plasma membrane is compromised.

To maximize the signal-to-noise ratio for the cisplatin assay, a rapid 1-minute “pulse” protocol was developed which allows cisplatin to bind to the abundant reaction sites exposed in a dead cell, while minimizing the amount of time that cisplatin would have to cross the plasma membrane of a live cell. Signal-to-noise ratios of greater than 10-fold separating live and dead cells were routinely observed (Figure 2–5). It may be even possible to identify other platinum compounds with lower membrane diffusion rates than cisplatin, which would further increase the signal-to-noise ratio. Although detectable amounts of cisplatin are bound to live cells (Figures 1–4), this may primarily be due to non-specific labeling including ionic adsorption to the plasma membrane, reaction with extracellular proteins, and diffusion across the plasma membrane.

The ability of cisplatin to detect dead cells is due to its access to the cell interior through the compromised plasma membrane, characteristic of dead cells, followed by rapid (minutes) formation of platinum-sulfur bonds with protein nucleophiles such as R-SH and R-SH-CH_3. This is in contrast to cisplatin’s covalent reaction with DNA purine residues which take considerably longer to occur (hours) and are associated with cisplatin’s cytotoxic effects(25). By contrast, in live cells cisplatin diffuses slowly across the intact plasma membrane and is minimally detected for the 1 minute duration of the assay.

Despite the documented induction of the cellular DNA damage response by prolonged cisplatin exposure(26), the “pulse” method described here did not induce an observable DNA damage response or pro-apoptotic signaling in KG-1 cells 6 hours after the cisplatin pulse (Figure 5).

Compared to other viability reagents currently available for mass cytometry, including the Rh- and Ir-intercalators(14,16) and Pd/Pt-chelated EDTA polymers, covalent modification of cellular components by cisplatin ensures stability of viability measurements when they are incorporated into cytometry protocols involving multiple rounds of washing and processing (Figure. 5). The stability of cisplatin will be particularly useful in cases where cells need to be fixed and frozen for shipment, or for prospective studies.

The generic cisplatin used in the experiments shown here was synthesized from naturally occurring platinum, which contains six stable isotopes (190, 192, 194, 195, 196, 198 A.M.U.). The platinum isotopes occupy mass channels, which, while within the range of the mass analyzer, have not been used by any of the metal-tagged antibodies in previous and ongoing studies (17,18). For the data shown in this study, viability determinations were based on measuring only the most abundant isotope (Pt195). Synthesis of cisplatin from isotopically enriched platinum would limit the detection spectrum to a single channel, and would allow for an equal signal at lower concentrations of cisplatin while freeing the other platinum channels for additional markers.

The quantification of cisplatin-labeled dead cells developed here for single cell mass cytometry, allows their removal from data analysis of any experimental system. The resultant data generated higher quality signal to noise and therefore support incorporating the cisplatin viability determination into mass cytometry protocols as routine.

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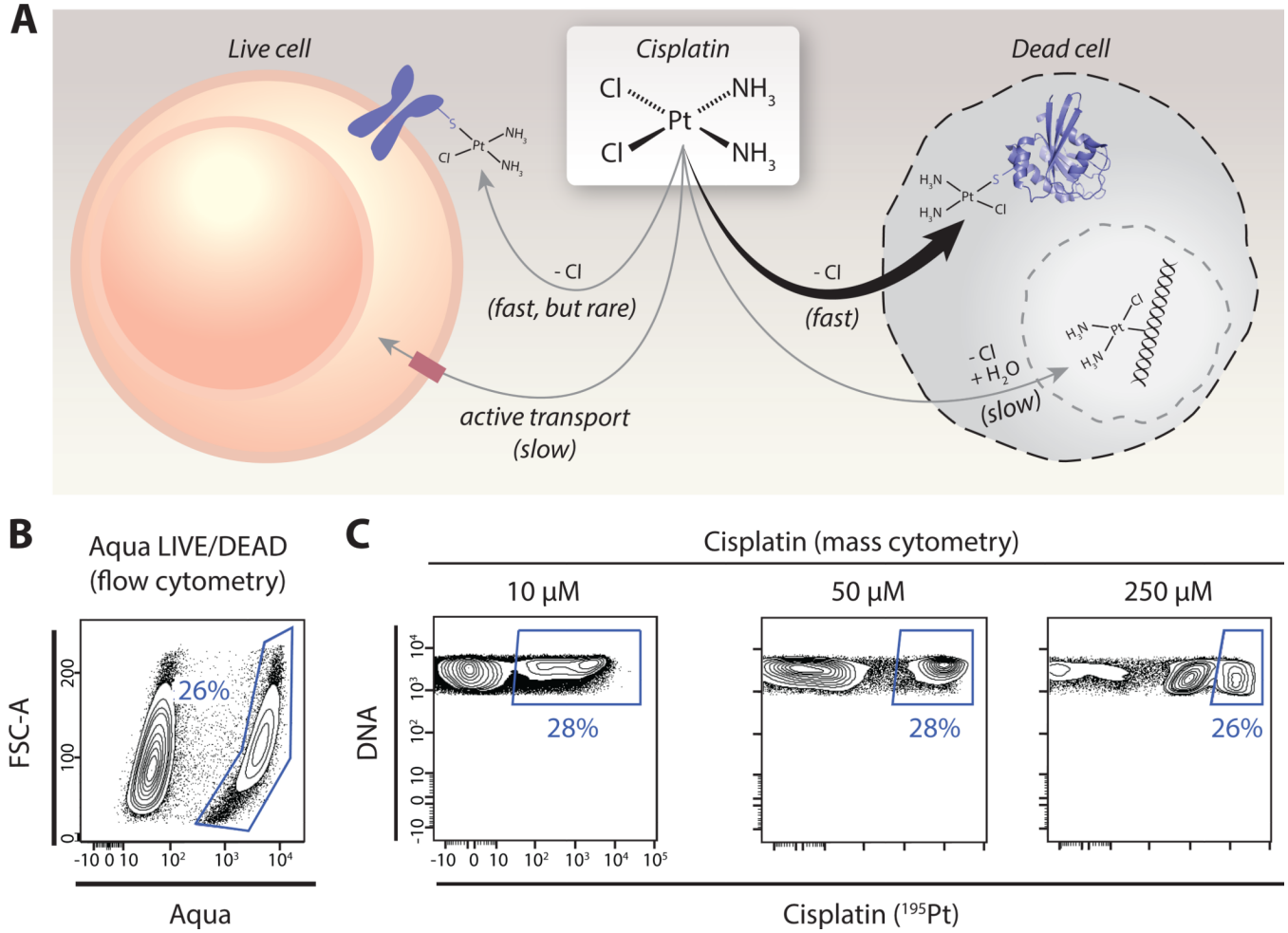

Figure 1.

Cisplatin as a viability reagent for mass cytometry. (A) In a one-minute "pulse" staining protocol, cisplatin preferentially reacts with protein in dead cells. (B) As a basis for comparison, an aliquot of partially heat-killed KG-1 cells was labeled with an amine-reactive fluorescent viability stain (Aqua). (C) Additional aliquots of partially heat-killed KG-1 cells were labeled with cisplatin at the concentrations shown and analyzed by mass cytometry.

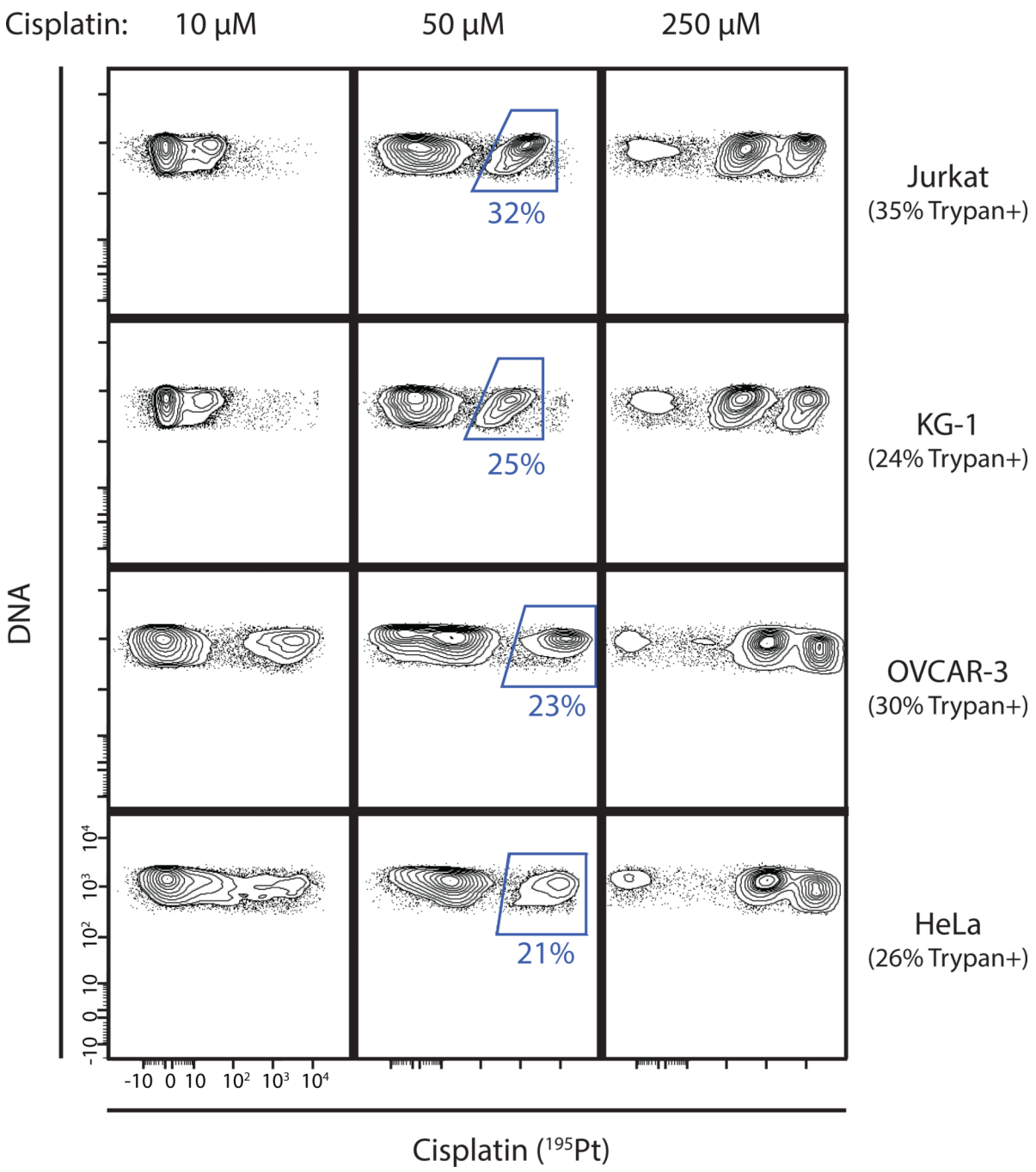

Figure 2.

Cisplatin as a viability indicator for suspension and adherent cell lines. Cisplatin viability labeling was performed at 10, 50 and 250 µM final concentrations for suspension (Jurkat, KG-1) and adherent (HeLa, OVCAR3) cell lines. For all cell lines the percentage of dead cells was comparable as determined by mass cytometry and Trypan blue exclusion.

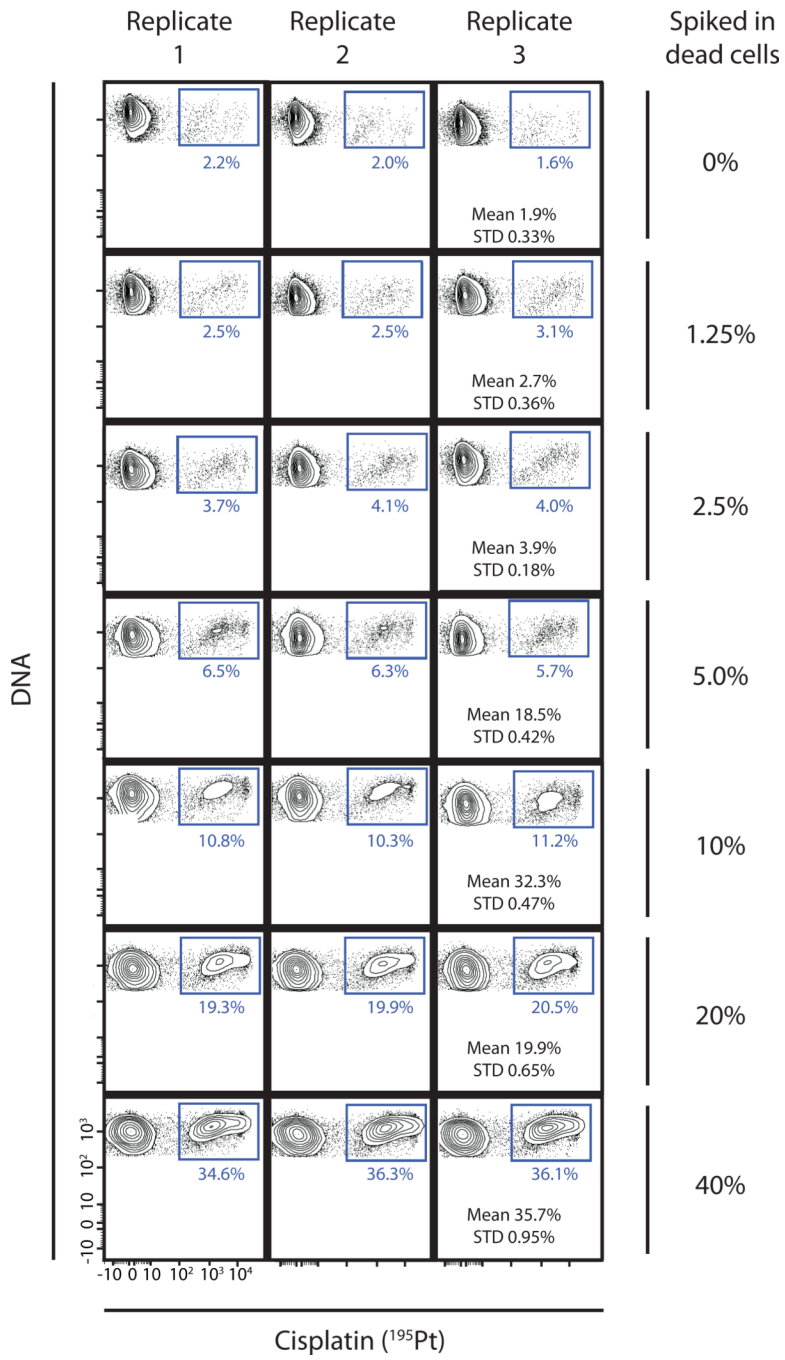

Figure 3.

Reproducibility and dynamic range of the cisplatin viability reagent by mass cytometry. Triplicate samples of HL-60 cells spiked with varying percentages of dead cells ranging from 0–40% were analyzed by mass cytometry using the cisplatin viability reagent. The average percent of dead cells in each sample with their standard deviation are shown. Mass cytometry measurements of cisplatin labeling reproducibly determined the percentage of dead cells over the whole analysis range.

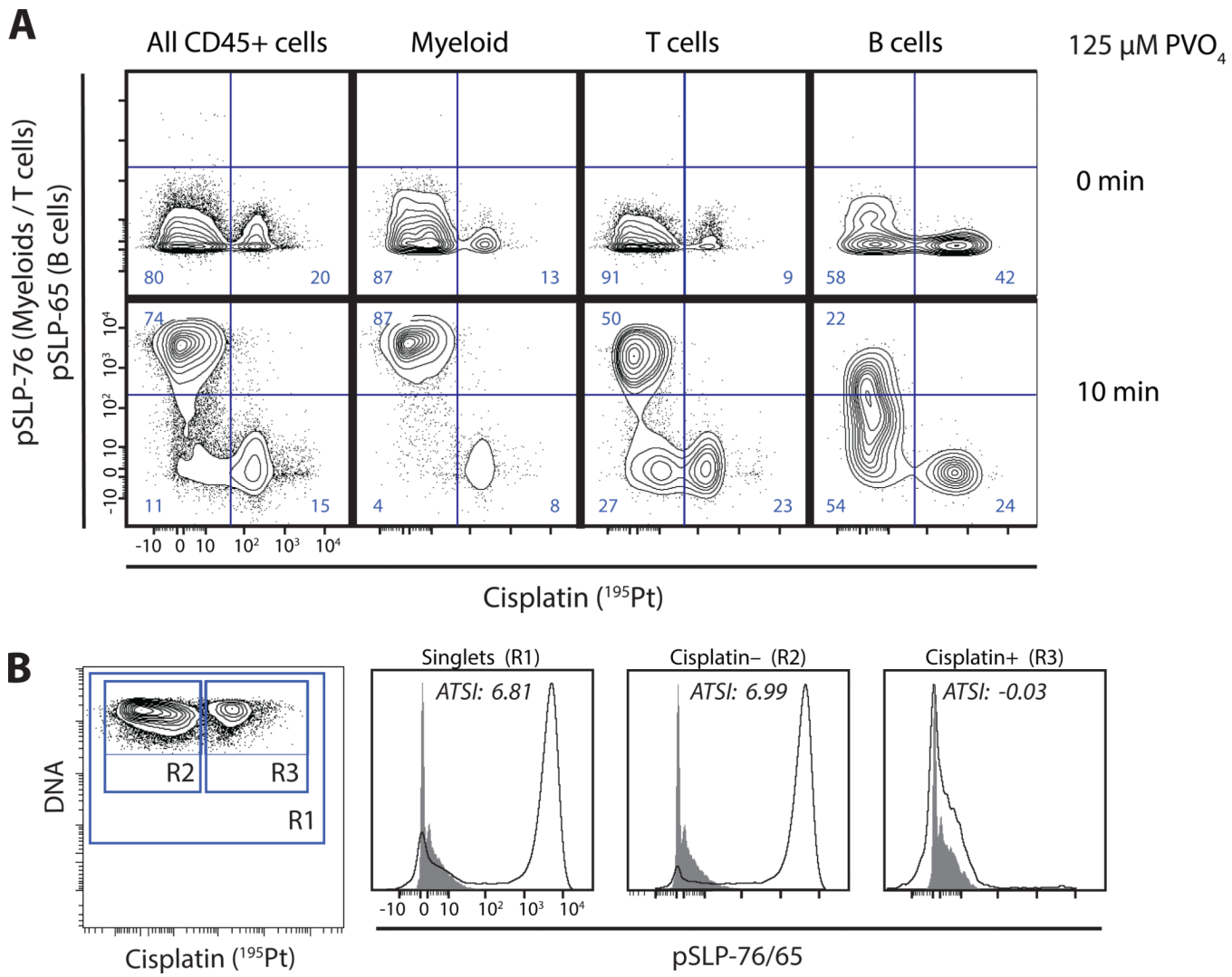

Figure 4.

Cisplatin-high PBMCs were unable to signal in response to pervanadate stimulation. (A) Cryopreserved peripheral blood mononuclear cells were labeled with cisplatin, 25 µM, final concentration for one minute and treated with PVO_4, 125 µM, final concentration for 15 minutes. An antibody that cross-reacted with phosphorylated SLP-76 (Tyr128) and SLP-65 (Tyr72) was used as an indicator of intracellular signaling. (B) Histogram overlays shown for pSLP-76/65 in untreated (grey) and vanadate-treated (white) populations. (R1) includes all cells, (R2) only cisplatin low and (R3) only the cisplatin high population.

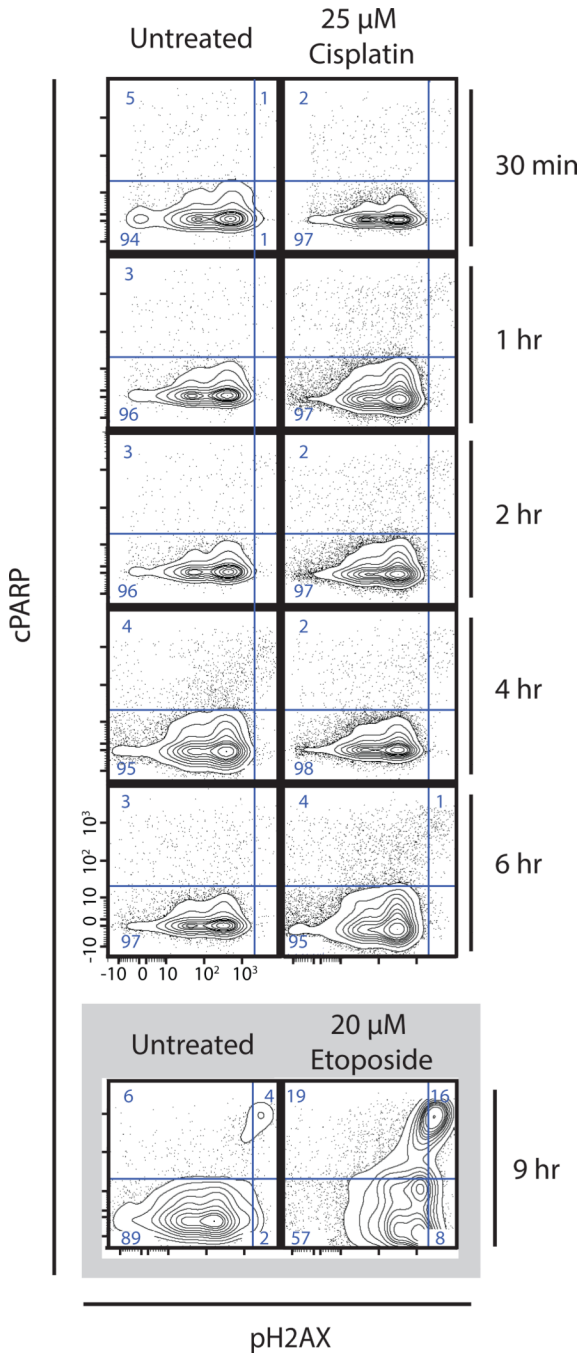

Figure 5.

Cisplatin viability labeling did not induce DDR or apoptosis. When compared with untreated cells, no induction of phosphorylated Ser139 on H2AX (pH2AX) or cleaved PARP (cPARP) was apparent in KG-1 cells incubated for 30, 60, 120, 240 and 360 minutes in cisplatin-free media following a 1-minute “pulse” treatment with cisplatin, 25 μM final concentration. A separate aliquot of cells was treated continuously with 20 μM etoposide for 9 hrs as a positive control for the pH2AX and cPARP antibodies.

Table 1

Antibodies used to analyze PMBCs.

Isotope	Antigen	Clone	Final concentration (μg/mL)	Supplier
Pr141	CD3	145-2C11	3	Invitrogen
Sm154	CD45	HI30	2	Biolegend
Sm147	CD20	H1	3	Biolegend
Gd158	CD33	WM53	3	Biolegend
Gd160	pSLP-76 (Y128) / pSLP-65 (Y72)	J141-668.36.58	1	BD

Table 2

Antibodies used to analyze DNA damage response in KG-1 cells.

Isotope	Antigen	Clone	Final concentration (μg/mL)	Supplier
Nd143	pH2AX(S139)	JBW301	1.2	Millipore
Er167	cleaved PARP	F21-852	1	BD