# *In Vitro* Drug Effects on Cancer Cell Morphology and Functional State Revealed by Multiparameter Imaging Mass Cytometry

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## Introduction

Results of *in vitro* cell culture drug testing are correlated with clinical response to chemotherapy: The benefit of using *in vitro* models lies in the ability to probe cellular response in a controlled closed system, where the effects of drug concentrations, treatment duration, drug efflux kinetics and multidrug combinations can be assessed using a variety of cell biology techniques. However, obtaining highly multiplexed data for surface, intracellular and functional markers for each cell within a test well has been challenging using standard immunofluorescence techniques.

Imaging mass cytometry (IMC) (1,2) allows us to investigate complex effects of chemotherapy drugs as well as the intracellular localization of metal-containing drugs (e.g., cisplatin and oxaliplatin), ruthenium-containing drugs (e.g., NAMI) and cytostatic agents (e.g., nocodazole and etoposide) (Table 2). A large panel of metal-tagged antibodies (Table 1) was used to analyze responses to drug treatments at the single-cell level. Proteins involved in DNA damage repair, apoptosis, cell proliferation, substrate adhesion, organelle morphology and signaling pathways, as well as surface and cytoskeletal markers, were studied. S-phase cells were visualized by detection of iodine ($^{127}$I) from 5-iodo-2′-deoxyuridine (IdU) incorporated by growing cells.

**Imaging mass cytometry workflow**. The immunostained and dried samples of tissue sections (FFPE or cryosections) and cells attached to glass slides are inserted into the ablation chamber of the Hyperion™ Imaging System (Fluidigm), where a 1 µm spot-size pulsed UV laser ablates the tissue. Isotopes associated with each spot are transferred into the mass cytometer, detected and indexed against the source location, yielding an intensity map of the target proteins throughout the tissue.

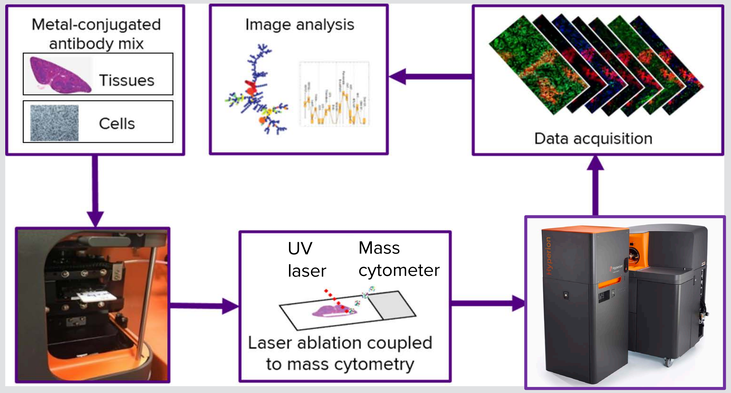



**Table 1. Metal-conjugated antibodies against human antigens**

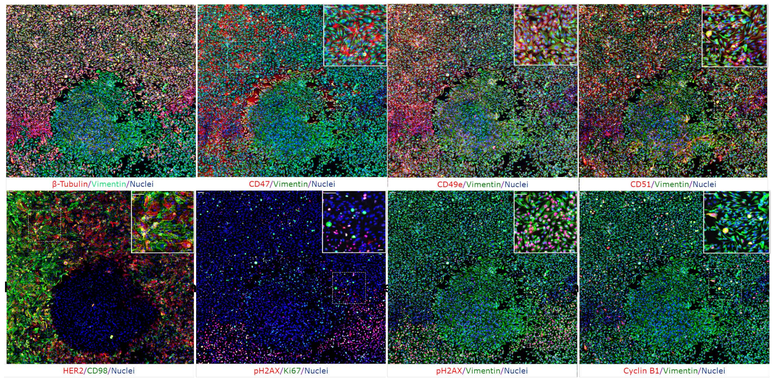
| Metal-conjugated antibodies to surface markers | Clone | Fluidigm/other vendor catalog number | Metal-conjugated antibodies to intracellular markers | Clone | Fluidigm/other vendor catalog number |
|---|---|---|---|---|---|
| B7-H3-$^{173}$Yb | D9M2L | CST™ 14058BF | Beta-Catenin-$^{147}$Sm | D10A8 | 3147005A |
| CD142-$^{144}$Nd | NY2 | BioLegend® 365202 | Beta-Tubulin-$^{171}$Yb | rabbit poly | Abcam ab6046 |
| CD24-$^{166}$Er | ML5 | 3169004B | CD107a-$^{151}$Eu | H4A3 | 3151002B |
| CD326-$^{141}$Pr | 9C4 | 3141006B | c-Myc-$^{176}$Er | 9E10 | BioLegend® 626802 |
| CD47-$^{209}$Bi | CC2C6 | 3209004B | cPARP(Asp214)-$^{140}$Nd | F21-852 | 3143011A |
| CD44-$^{166}$Er | BJ18 | 3166001B | CyclinB1-$^{156}$Dy | GNS-1 | 3164010A |
| CD49e-$^{160}$Gd | HI10a | 3156001B | Histone3-$^{176}$Yb | DIH2 | 3176016A |
| CD51-$^{169}$Tm | NY2 | BioLegend 327902 | Keratin (CK8/18)-$^{174}$Yb | CS1 | 3174014A |
| CD59-$^{173}$Yb | p282 (H19) | 3173009B | Ki67-$^{168}$Er | B56 | 3168007B |
| CD61-$^{12}$$^{1}$Nd | VI-PL2 | 3150001B | p53-$^{150}$Nd | DO-7 | Abcam ab80644 |
| CD71-$^{175}$Lu | OKT-9 | 3175011B | Pan-Keratin-$^{162}$Dy | C11 | 3162027A |
| CD9-$^{172}$Yb | SN4 C3-3A2 | 3171009B | pH2AX(pS139)-$^{165}$Ho | N1-431 | BD Biosciences 560443 |
| CD98-$^{159}$Tb | H4A3 | 3151002B | pHistone3-$^{175}$Lu | Ser28 (HTA28) | 3175012A |
| ErbB2/HER2-$^{153}$Eu | 24D2 | BioLegend 324402 | Thioredoxin-$^{146}$Nd | 2G11/TRX | 3146016B |
| EGFR-$^{170}$Er | AY13 | 3170009B | Vimentin-$^{154}$Gd | RV202 | 3156023A |
| | | | Intercalator-103Rh | N/A | 201103B |
| | | | Intercalator-Ir | N/A | 201192B |
| | | | IdU | N/A | 201127 |

**Table 2. Drugs and cell lines**

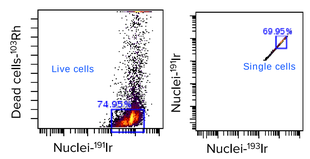
| Drug | Amount | Drug treatment | Drug effect | Cell line tested |
|---|---|---|---|---|
| Cisplatin | 5 µM | 24 hours | DNA damage | A-431 (ATCC® CRL-1555™) Skin epidermoid carcinoma |
| Cisplatin | 20 µM | 24 hours | DNA damage | HeLa (ATCC CCL-2™) Cervix adenocarcinoma |
| NAMI | 25 µM | 2 hours | Migration and adhesion inhibitor, cell cycle arrest, induction of apoptosis | A-431 (ATCC CRL-1555) Skin epidermoid carcinoma |
| Etoposide | 10 µM | 18 hours | DNA damage | HeLa (ATCC CCL-2) Cervix adenocarcinoma |
| Nocodazole | 10 ng/mL | 24 hours | Cell cycle arrest in mitosis | HeLa (ATCC CCL-2) Cervix adenocarcinoma |

## Results

### IMC false-color images and quantitative analysis of SKOV3

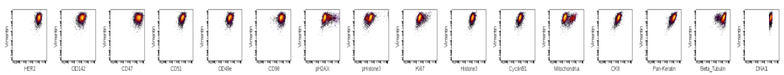


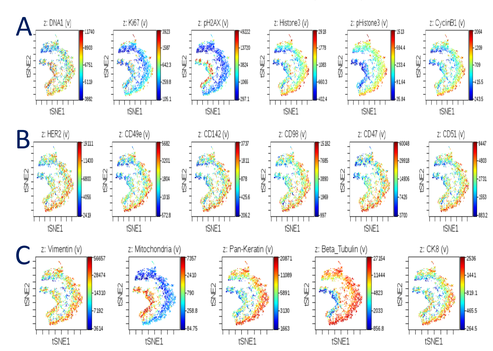

Cells were grown on chamber slides (Lab-Tek™ II Chamber Slide™) for several days and stained with a mixture of metal-conjugated antibodies. SKOV3 cell culture 16-bit TIFF images were generated from raw IMC files (MCD files). Scale bar = 100 µm. Nuclei were stained with DNA-binding Ir-intercalator. The Ir-intercalator DNA $^{191/193}$Ir dot plot shows correlation of the intensity of the two channels as a red density of points corresponding to single cells.




**Quantitative image analysis**

Marker quantitation from the ablation area was done with the help of the open source bioimaging platform Icy (3) and the 16-bit digital RGB IMC TIFF images. By using a segmentation method called K-means color quantization, we processed and extracted one iridium nuclear binary mask generated as a TIFF image, which was then used for segmentation of the raw data by a segmentation algorithm in Mathematica® software.



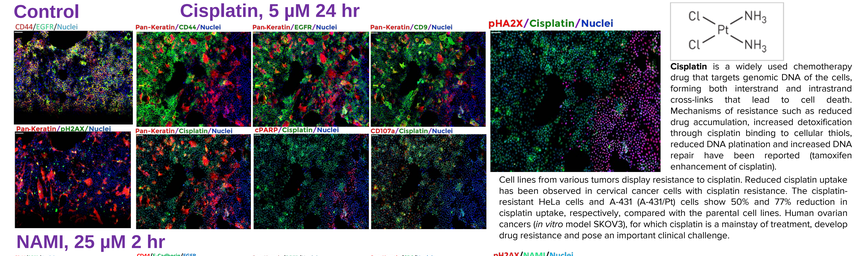




**SKOV3 cell heterogeneity analyzed by viSNE: (A) nuclear markers, (B) surface markers, (C) cytoplasmic markers**

4,229 segmentation areas corresponding to single cells were converted into an FCS file with mean values per object for each channel. 4,000 single cells were mapped to a two-dimension viSNE map, level of expression of each marker is shown using a heat map gradient. Analysis of the FCS file generated from raw data (not normalized) is then performed using the Cytobank platform. Using single-parameter histograms, correlative dot plots and viSNE analysis (4), we can map the multiparametric data from an IMC region into different formats.

### *In vitro* drug testing on adherent cell lines

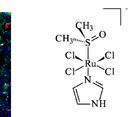
**A431 cell culture**




Cell lines from various tumors display resistance to cisplatin. Reduced cisplatin uptake has been observed in cervical cancer cells with cisplatin resistance. The cisplatin-resistant HeLa cells and A-431 (A-431/Pt) cells show 50% and 77% reduction in cisplatin uptake, respectively, compared with the parental cell lines. Human ovarian cancers (*in vitro* model SKOV3), for which cisplatin is a mainstay of treatment, develop drug resistance and pose an important clinical challenge.

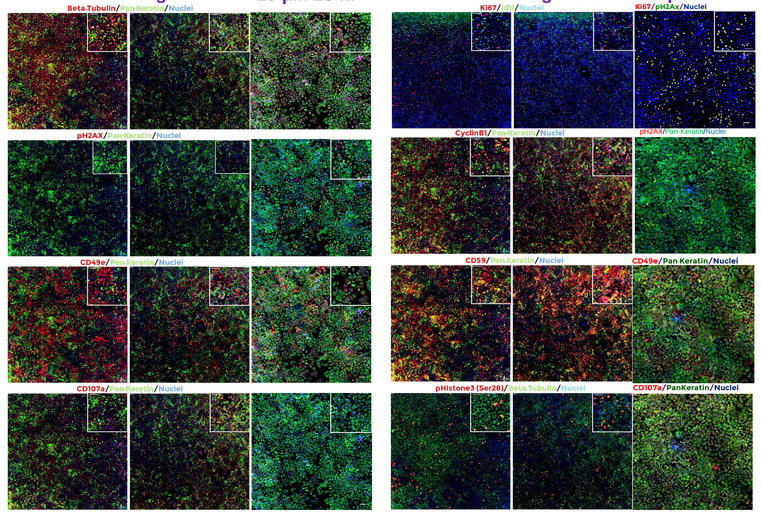


**Cisplatin** is a widely used chemotherapy drug that targets genomic DNA of the cells, forming both interstrand and intrastrand cross-links that lead to cell death. Mechanisms of resistance such as reduced drug accumulation, increased detoxification through cisplatin binding to cellular thiols, reduced DNA platination and increased DNA repair have been reported (tamoxifen enhancement of cisplatin).



**NAMI-A** is a ruthenium anticancer agent. NAMI is an acronym for new antitumor metastasis inhibitor. Ruthenium, unlike traditional platinum complexes such as cisplatin, shows greater resistance to hydrolysis. NAMI-A doesn't inhibit primary tumor growth but decreases the speed at which metastasis occurs. It has also been found that when compared to highly cytotoxic platinum compounds, NAMI-A is much less cytotoxic and has a different mechanism of action.

**HeLa cell culture**




**Etoposide** is a topoisomerase inhibitor. Etoposide forms a ternary complex with DNA and topoisomerase II enzyme, prevents re-ligation of the DNA strands and by doing so causes DNA strands to break. Cancer cells rely on this enzyme more than healthy cells, since they divide more rapidly. This causes errors in DNA synthesis and promotes apoptosis of cancer cells. Etoposide is used as a form of chemotherapy for cancers such as sarcomas, lung, testicular cancers, lymphoma and nonlymphocytic leukemia and glioblastoma.

**Nocodazole** is an antineoplastic agent that exerts its effect in cells by interfering with the polymerization of microtubules. Nocodazole stimulates the expression of LATS2, which potently inhibits the Wnt signaling pathway. Microscopy of nocodazole-treated cells shows that they do enter mitosis but cannot form metaphase spindles because microtubules (of which the spindles are made) cannot polymerize.

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