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# Utilizing inherent fluorescence of therapeutics to analyze real-time uptake and multi-parametric effector kinetics

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ABSTRACT

The precise detection of pharmaceutical drug uptake and knowledge of a drug's efficacy at the single-cell level is crucial for understanding a compound's performance. Many pharmaceutical drugs, like the model substances Doxorubicin, Mitoxantrone or Irinotecan, have a distinctive natural fluorescence that can be readily exploited for research purposes. Utilizing this respective natural fluorescence, we propose a method analyzing simultaneously in real-time the efficiency, effects and the associated kinetics of compound-uptake and efflux in mammalian cells by flow cytometry. We show that real-time flow cytometric quantification of compound-uptake is reliably measured and that analyzing their respective uptake kinetic provides additional valuable information which can be used for improving drug dosage and delivery. Exploiting the native fluorescence of natural compounds is clearly advantageous compared to the usage of non-related fluorescent uptake-reporter substances, possibly yielding in unphysiological data. Flow cytometric analysis allows live-dye based multi-parametric high-throughput screening of pharmaceutical compound activity, improving cytotoxicity testing by combining several assays into a single, high resolution readout. This approach can be a useful tool identifying potential inhibitors for multiple drug resistance (MDR), representing a major challenge to the targeted treatment of various diseases.



## 1. Introduction

Many pharmaceutical agents require intracellular presence to exert their therapeutic potential [1]. Consequently, these substances must pass through the cell-membrane by passive diffusion or via carrier-mediated transport. The complex procedure of intracellular accumulation differs among drug types, and important factors include uptake, retention, distribution in, and efflux from the cell [2]. A more thorough understanding of the associated steps and their respective kinetics would provide a clear advantage in drug identification, design, dosing and delivery, and could lead to improved efficiency in the lead compound identification process. More than 70% of all cytostatic small molecules used in cancer chemotherapy are natural products or directly derived there from [3]. As such, they exhibit complex structures with many delocalized electrons and many, for example Doxorubicin [4] and Camptothecin [5], possess inherent fluorescence, a valuable trait most often neglected. Current methods in the analysis of drug uptake include assays using radioactively labeled compounds [6], limited fluorescence microscopy [2] or measuring residual amounts of drug remaining in the supernatant after cell treatment [7]. These assays, besides being labor-intensive and time consuming, measure uptake indirectly using multiple samples in order to gain a limited endpoint-based kinetic analysis precluding any kind of multi-parametric co-analysis of effects caused by drug uptake.

In this study we describe protocols for measuring real-time uptake kinetics of several drugs using the advantage of sufficient intrinsic fluorescence of the actual compounds in order to perform flow cytometry allowing direct, single cell quantifications. Furthermore we show that additional live functional assays can be easily coupled with drug uptake analysis and that flexible simple dye based analysis of multiple drug-dependent parameters at the same time is possible. Due to this, the presented method is advantageous as it combines multiple recommended cytotoxicity assays [8] into a single experimental setup and actually allows direct correlation of uptake efficiencies with drug efficacy. Furthermore, screening for drugs exhibiting inherent fluorescence can be easily performed by multi-parametric flow cytometry and hence the presented method has additionally the potential to be applied in a number of pharmaceutical areas including high-throughput screening in drug discovery and development.

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## 2. Material and methods

### 2.1. Drugs

Doxorubicin, Etoposide, Irinotecan, Methotrexate, Mitoxantrone and Topotecan were provided by University Medical Center of the Johannes Gutenberg University, Mainz. All chemicals were dissolved in PBS (Invitrogen, Germany) at concentration of 100 μM and stored away from light at 4 °C for a maximum of 3 days.

### 2.2. Cell culture

U937 cells, a human monocytic leukemia cell line, were obtained from the German Cancer Research Center (DKFZ, Heidelberg, Germany). Cells were cultured in complete RPMI 1640 medium with 2 mM L-glutamine and phenol red indicator (Invitrogen, Germany) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Germany) and 1% penicillin (100 U/ml)- streptomycin (100 μg/ml) (Invitrogen, Germany) in T75 culture flasks (Thermo Scientific, Germany). Cells were maintained in a humidified environment at 37 °C with 5% $CO_2$. They were sub-cultured twice per week with an initial cell density of $2 \times 10^5$ c/ml. Final cell density did not exceed $2 \times 10^6$ c/ml. Cells were passaged to a maximum of 10 weeks.

### 2.3. Measuring quantitatively inherent fluorescent drug uptake in live cells by flow cytometry

U937 cell suspension was transferred to a 50 ml centrifuge tube (Sarstedt, Germany) and centrifuged at 1200 rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended in RPMI 1640 medium with 2 mM L-glutamine containing no phenol red (Invitrogen, Germany) to reach a final cell concentration of $5 \times 10^5$ cells/ml. Two milliliters of the cell suspension were then transferred into 5 ml FACS tubes (Becton–Dickinson, Germany). The cells were measured on a LSR-Fortessa FACS analyzer (Becton–Dickinson, Germany) with excitation and emission parameters as shown in Table 1. The background fluorescence of the cells was measured for 2 min and adjusted to be 10× above the electronic noise of the analyzer to ensure precise measurements even with large cellular based coefficient of variation (CV). Dead cells were excluded either by staining with 2 μM Hoechst (Merck, Germany) or by FSC/SSC gating which in most cases was sufficient. After 2 min the FACS tube was removed from the flow cytometer without stopping the recording and the appropriate drug was added immediately in the desired concentration (See Figs. 1–3). The cell suspension containing the drug was gently but thoroughly mixed and reinserted into the flow cytometer within 30 s after removing it from the machine. The measurements continued up to 20 min total time at a flow rate of 50–70 cells per second while recording the area signal of all respective channels. Cytographs were analyzed using FlowJo software (Celeza, Switzerland).

### 2.4. Correlating direct fluorescence with supernatant fluorescence reduction for direct quantification of drug uptake

In order to correlate FACS measured fluorescence with physical drug uptake a standard curve of cellular uptake of Doxorubicin into U937 cells was ascertained fluorometrically based on the residual amounts of Doxorubicin fluorescence in the supernatant of the measured cultures. In detail, $1 \times 10^6$ U937 cells in RPMI 1640 medium without phenol red indicator were transferred into a FACS tube in a total volume of 2 ml. Doxorubicin was added in various concentration (0.3, 0.6, 1.2, 2.4, and 4.8 μM) and the cells were incubated for 20 min. After incubation, the cells were transferred to a 15 ml centrifuge tube (Sarstedt, Germany) and centrifuged at 1200 rpm for 5 min. One hundred microliters of the supernatant was transferred into each of three wells of a white 96-well plate (Thermo Scientific, Germany). The residual fluorescence in the supernatant was measured at an excitation wavelength of 485 nm and an emission wavelength of 590 nm using a TECAN Infinite M200 pro plate reader. Controls included FACS tubes with 2 ml of conditioned RPMI 1640 medium without cells that had been incubated with appropriate concentrations of Doxorubicin. The percent cellular drug uptake relative to reference solutions was calculated and used to calculate the μM Doxorubicin taken into the cells.

The drug uptake measured from the different supernatants was correlated with the background fluorescence increase (GeoMean Gate 3 – GeoMean Gate2) of the corresponding samples measured by FACS. The amount of internalized Doxorubicin of each sample was converted to ng and divided by the background subtracted fluorescence increase of the corresponding sample, resulting in case of Doxorubicin in a very exact correlation of ~0.5 ng Doxorubicin per fluorescence intensity unit (FIU). This correlation was plotted using Excel and is presented in Fig. 1H.

### 2.5. Multiplexing of drug uptake and functional screening for ROS induction, DNA integrity and live/dead cell ratio in live cells

2′,7′-Dichlorodihydrofluorescein diacetate ($H_2$DCFH-DA) (Sigma–Aldrich, Germany) is a probe used for the highly sensitive and quantifiable detection of reactive oxygen species (ROS). The non-fluorescent $H_2$DCFH-DA diffuses into the cells, it is cleaved by cytoplasmic esterases into 2′,7′-dichlorodihydrofluorescein ($H_2$DCF) and is thereby trapped in the cell. In the presence of hydrogen peroxide, $H_2$DCF is oxidized to the fluorescent molecule dichlorofluorescein (DCF) by peroxidases. The fluorescent signal emanating from DCF can be measured and quantified by flow cytometry, thus indicating the concentration of ROS present in the cell [9,10].

In detail, $2 \times 10^6$ U937 cells were transferred into a 15 ml centrifuge tube (Sarstedt, Germany) and washed with 2 ml of PBS (Invitrogen, Germany) by centrifugation at 1200 rpm for 5 min. Subsequently, cells were suspended in 2 ml of PBS containing 2 μM $H_2$DCFH-DA and incubated for 20 min in darkness. After incubation, cells were washed with 2 ml of PBS by centrifugation at 1200 rpm for 5 min and suspended in 2 ml phenol red-free RPMI 1640 medium containing 4.8 μM Doxorubicin. In other cases the cells were incubated with Doxorubicin for 16 h in clear media prior to staining with $H_2$DCFH-DA. $H_2$DCFH-DA loaded cells in culture medium without Doxorubicin were used as untreated control. After 3 h incubation with Doxorubicin, cells were washed with 2 ml of PBS by centrifugation at 1200 rpm for 5 min and suspended in 2 ml phenol red-free RPMI 1640 medium. In the case of multiparametric analysis being performed, the cells were incubated 16 h with Doxorubicin and additionally incubated with 1 μl Vybrant DyeCyle reagent and 1 μl Sytox (both Life Technologies, Germany) per ml of $1 \times 10^6$ cells/ml cell suspension for 30 min at 37 °C. Subsequently cells were measured in a LSR-Fortessa flow cytometer. Doxorubicin was measured with 561 nm excitation (150 mW) and detected using a 610/20 nm bandpass filter; DCFDA was measured with 488 nm excitation (100 mW) and detected using a 530/30 nm bandpass filter; SytoxRed was measured with 640 nm excitation (40 mW) and detected using a 670/30 nm bandpass filter; Vybrant DyeCycle DNA dye was measured with 405 or 355 nm excitation (100/20 mW), in both cases using a 450/50 nm bandpass filter. All parameters were plotted on a logarithmic scale except for the Vybrant DyeCycle, as it was used for cell cycle analysis and henceforth was plotted linearly. All cells for live multi-parametric assays were gated exclusively on the criteria of being single cells using Area vs Width scattering of the side-scatter