Heterogeneous cellular drug uptake in chemotherapy

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Introduction

Heterogeneous cellular drug uptake exists in chemotherapy [1, 2]. This phenomenon is related to the different rates of cells growth and gradient regions of hypoxia and acidity of tumor microenvironment [3]. Besides, the distribution of many drugs within tumors is also heterogeneous [3]. All these factors can affect cancer cell sensitivity to drug treatment [4, 5]. Heterogeneity in drug uptake and cellular sensitivity would lead to fractional killing of cancer cells and failure of chemotherapy [6, 7]. Individual cells behave variously in resistance to a specific drug could also lead to acquired multidrug resistance (MDR) [8, 9]. Thus, heterogeneous cellular drug uptake has a profound influence on the treatment of diseases. Herein, we review the heterogeneous cellular drug uptake in chemotherapy, and will focus on analytical methods for detection of the heterogeneity and its effects on cancer treatment.

Single cells analysis

Heterogeneity is an inherent character of cell populations [10]. However, traditional researches usually relay on bulk methods to determine the average protein or biomolecular content of a large number of cells. The mean values derived from these approaches may reflect very different underlying population distributions and mask cell-to-cell variation [8, 11]. Therefore, techniques are needed to directly measure cellular content at the single-cell level and yield the actual distribution of particular content in a population of cells.

Initially, flow cytometry [12] and microscopy [13] are commonly used for single cells detection. They are...
convenient methods for detection of large number of single cells, but with limitations of lack of sample separation capacity and poor quantitative measurement. During the last two decades, various analytical methods were developed for chemical single cells analysis, which lyse the cells individually and separates, characterizes and quantifies a large number of components in single cells [14, 15]. Microelectrophoretic separations, using capillary electrophoresis [16] or microfluidics [17], are suitable for manipulating the ultra-small size of single cells and presented as the main analytical methods. Hundreds of intercellular constituents, including ions, DNA, amino acids, proteins, small molecules, organelles, et al are successfully detected and large chemical information from single cells are obtained [14-17].

Analysis of drug uptake in single cells and its heterogeneity

Although innovations in the instrumentation and sampling methods have extensively widen the range of analytes and cell types, the majority of researches are on the intracellular components. Compounds such as drugs from extracellular circumstance are rarely touched upon. A few works on analyses of drug uptake in single cells will be discussed and be sorted by analytical methods.

Flow cytometry

Flow cytometry (FCM) utilizes optical detection for both physical and chemical characters of single cells in a flow stream. Physical properties include cell size and sort which can be measured by different angle of scatter light. Chemical compositions in a single cell are examined by intrinsic fluorescence of the components or using fluorescent reagents for cells labeling [18].

Drugs with intrinsic fluorescence used in chemotherapy, such as anthracyclines, can be directly detected in a large number of single cells. For example, the uptake of doxorubicin (DOX) in individual cells has been studied by FCM [1, 2, 12, 19-22]. Fluorescence profiles of cellular heterogeneity and different patterns of DOX uptake were observed in both cultured and patients’ cancer cells [1, 2, 12, 21, 22]. However, FCM solely provides relative fluorescent intensity of intracellular drug and cannot reflect the real drug content [20]. Further, the magnitude of the cellular heterogeneity in drug uptake was roughly described by fluorescence intensity [2, 21, 22], histograms distribution [12], or the percentage of cells with detectable drug fluorescence [1].

Image-based assay

In the early days, light microscopy was used to track the intracellular accumulation of fluorescent drugs at single cells level [13]. Lately, more powerful analytical tools are immerging to detect drugs uptake in single cells, such as confocal microscopy [23], X-ray nanoscopy and two-photon microscopy [24], et al. By image cytometry [14], more precise images are obtained of individual cells, and the dynamics of drugs uptake are recorded by serial images at different time intervals [23, 24]. However, the majority of researches emphasized on overall intracellular drug accumulation, not so many research looked into the heterogeneity in cellular drug uptake.

Thanks to the inventions in techniques and the aware of cellular heterogeneity, more advanced systems are used to
track drugs uptake in single cells and the variation in cells response. Using high-throughput automated time-lapse light microscopy \cite{4, 5}, the same cell lines treated with the same or different line of drugs displayed profound variation in fate profiles and proteomics. High-content image based assays \cite{25} facilitated the quantification of cellular phenotypes to characterize patterns of heterogeneity observed within cellular populations. Notably, image assays developed more slowly than FCM because it lacks of optical and data manipulation tools \cite{14}.

**Capillary electrophoresis**

Since 1990’s, capillary electrophoresis (CE) is extensively utilized for analysis of chemical components in single cells \cite{15, 16}. This method is also termed as chemical cytometry \cite{14}. Because the inner diameter of capillary can be 15-50 μm, it is particularly suitable for sampling and separating the cellular compositions of single cells in the microchannel of a capillary. Meanwhile, CE can coupled with laser induced fluorescence (LIF) detection with detection limits as low as 10⁻¹⁸ to 10⁻²¹ mol, which also facilitate the chemical detection of single cells.

Few works are on analysis of drugs uptake in single cells by CE. DOX in single NS-1 mouse hybridoma cells \cite{26}, DOX contents in single nuclei \cite{27} or acidic organelles \cite{28} of single human leukemia cells were detected by Arriaga’s group using CE-LIF. Uptake and metabolism profiles of single cells are profoundly different from cells population, as well as between each other’s. However, not a quantitative study of cellular variation in drug uptake was carried out until then.

Our group utilized CE-LIF \cite{29} not only to identify and quantify the DOX uptake in single cancer cells depending on drug concentration and exposure time, but also quantify the cellular heterogeneity in drug uptake using relative standard division (RSD) of drug uptake amount. Results showed that marked heterogeneity in drug uptake exists among K562 cells. This cellular heterogeneity in DOX uptake decreased with the increasing concentration of DOX, and then reached equilibrium. On the other hand, it showed no patterns according to the drug exposure time.

**Microfluidics**

Due to the complex and laborious manipulation of single cells, one limitation of CE used for chemical cytometry is low throughput, typical 3-5 cells h⁻¹, or less than 40 cells per day \cite{15}. On the other hand, micro-fluidic platforms \cite{17, 30} with micrometer channel integrate multiple operations of single cells on one chip, showing great compatibility and possibility for chemical analysis of single cells.

Recently, our group invented a novel polydimethylsiloxane (PDMS) microfluidic chip to fast detection of DOX uptake and membrane surface P-glycoprotein (P-gp) expression in single human leukemia K562 cells \cite{31}. The analytical throughput was greatly improved based on continuous cell sampling, lysis and detection of intracellular contents in a flowing stream. The average throughput for analysis was 6-8 cells min⁻¹, much higher than that of CE-LIF method. Cells showed heterogeneous DOX uptake. Meanwhile, the cellular heterogeneity in DOX uptake decreased with the increasing drug concentration. These data agreed well with our previous CE-LIF results \cite{29}, indicating the feasibility and simplicity of the microfluidic chip for chemical cytometry.

By using microfluidic-based same-single-cell analysis (SASCA) \cite{32, 33}, Paul’s group investigated real-time drug transport kinetics of single cells and cellular differences-in response to drug efflux modulation. This technique may look into the drug resistance in cell subpopulations and be used as a prognostic method.

**Mass spectrometry**

Similar to antibodies used in fluorescence FCM, mass cytometry \cite{34} utilizes metal ion tags to label antibodies rather than fluorescence dyes. Single cells with isotope conjugates were sprayed as droplets into an inductively coupled argon plasma (ICP) and ionization of its atomic constituents were introduced into a time-of-flight (TOF) mass spectrometer. This technology dramatically increases the number of parameters (up to 40) that can be tested simultaneously in single cells. The atomic mass spectrometry does not require a compensation to correct the spectral overlap of fluorescence, as well as has no cell-dependent background signal. This analysis observed type-specific drug responses of human bone marrow cells \cite{35}. Besides, bismuth antiulcer drug uptake in single Helicobacter pylori cells was tracked by time-resolved ICP-MS \cite{36}. Both the ²⁴Mg and the ²⁰⁹Bi spike intensities were in large fluctuation, representing different growth conditions of cells. Mass spectrometry provides new directions in using time-resolved ICP-MS for analysis of metallodrug uptake at single-cell level.

**Effects of heterogeneous drug uptake on chemotherapy**

The fact that profound phenotypic heterogeneity exists in cellular populations has been widely observed and recognized. These variations bring genetically identical cells respond very differently to drugs even in the same environment \cite{4, 5}. However, does cellular heterogeneity contain biologically (or clinically) important information, and how it may impact pharmacology and the treatment of human disease are poorly understood \cite{25, 37}.

To try to address these challenging questions, our group made use of different schedules of DOX to treat cultured human cancer cells as a mode, and CE-LIF as the detection
method. We quantitatively investigated the variation of DOX uptake in single cells, and its corresponding pharmacological effect on cancer cells [38]. To our surprise, the cellular heterogeneity in DOX uptake was negatively correlated to DOX cytotoxicity, as well as surface P-gp expression in both K562 and MCF-7 cells (Fig. 1, \( r = -0.7680 \) to \(-0.9587\)). Meanwhile, quantitative regression equations of heterogeneous drug uptake effects were obtained by linear regression calculation.

This research showed that the reduced cellular variation in drug uptake could improve the outcome of chemotherapy [38]. The results agreed quite well with the clinical study of patients’ disease outcome nearly 30 years ago [1], but with more precise and quantitative data. Besides, the cellular difference in drug uptake may be useful in prediction of drug responses [38]. Meanwhile, combined chemotherapy that reduces the fraction of non-responders, or targets the proteins contributing most to variability in response, can be examined as a treatment strategy [37].

A lot of future works of this research can be considered. The investigation carried out on mice or other animal modes may provide more reliable results than cultured cell lines. More efficient analytical method, such as microfluidic chip or image-based systems will provide more single cells data of statistical significance. Advances in mathematical modeling methods and interpretation of patterns of cellular heterogeneity will be very useful in the development of treatments of human diseases.

Conclusions
The phenomena of cell individuality and cellular heterogeneity in drug uptake and responses in chemotherapy are gradually recognized and noticed. The reduced cellular variation in drug uptake could lead to higher drug cytotoxicity and a better outcome of chemotherapy. Researches demonstrated the significance of quantitative study of cellular heterogeneity in drug uptake, chemotherapy response and its full impact on pharmacology and treatment of human disease. The interpretation of cellular heterogeneity in drug uptake has large potential for applications in drug schedule regulation, outcome prediction, treatment modulation, and individual chemotherapy.

Conflict of interests
The authors declare that they have no competing interests.

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