論　文　内　容　要　旨

Single-Cell Screening of Tamoxifen Abundance and Effect Using Mass Spectrometry and Raman-Spectroscopy

（質量分析とラマン分光法によるタモキシフェンの量と効果の一細胞スクリーニング）

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Cells are known to be inherently heterogeneous. This means that their response to external stimuli like drugs varies from one cell to another. Despite this fact, current drug discovery studies are done using ensemble-averaged measurements of cell populations. These measurements obfuscate any information about potential subpopulations, as well as single-cell variation. This information loss was cited as one of the reasons for the failure of phase II clinical trials of the drugs. Therefore, it's essential that cell heterogeneity is accounted for in future drug studies. To achieve this, the plethora of cellular interactions between the cell and the drug must be accurately measured on the single-cell level (i.e. Drug-uptake, drug-metabolism, and drug-response).

To achieve this, I developed an integrated Raman spectroscopy and mass spectrometry (MS) platform. By integrating the two approaches into a cohesive platform, untargeted analysis of the drug response can be done using Raman spectroscopy, with the added possibility of quantifying the drug-uptake, and metabolism using mass spectrometry for drug-treated cells that were previously measured by Raman. The possible correlation between the drug-effect, and the drug or its metabolites' concentration can also be investigated on the single-cell level.

In this study, HepG2 cells were treated with the anti-cancer drug, tamoxifen. Tamoxifen is metabolized by HepG2 cells into its pharmacologically active metabolite 4-Hydroxy-tamoxifen (4-HT). It also has a toxic effect on the cell. HepG2 cells were divided into two groups, treated and untreated cells. Treated cells were incubated with 10μM tamoxifen dissolved in DMSO for 24 hours, while untreated cells were incubated with a corresponding volume of DMSO as control. The experiment was repeated for three consecutive days. In order to minimize growth phase variations, a pilot study was done to synchronize the growth of cell populations across the three experiments.

Cells were cultured in glass-bottom grid dishes, which allowed us to perform Raman measurements on a given cell while recording its location, followed by sampling the same cell using a micromanipulator for subsequent MS analysis. Raman measurements were performed on individual cells in single-line exposure mode, with 10 seconds exposure time. In each experiment, treated (80 - 120 cells) and untreated (35- 40 cells) cells were sampled, in total, 295 treated and 115 untreated cells were measured by Raman. Among these cells, 53 cells of similar dimensions were chosen for MS analysis. Hollow platinum coated glass capillaries with bore-size of 2-4 μm attached to a micromanipulator were used to sample the cells after Raman measurements. Each cell was sampled, then the capillary was disconnected from the micro-manipulator, and the organic solvent was added to the other end of the capillary. Finally, the capillary, along with its contents was attached to the nano-spray source of the MS instrument (Thermo's Q-Exactive) where single-cell MS measurements were done.

Raman measurements revealed spectral differences between treated and untreated cells which indicates that there are metabolic changes caused by the drug in the cells, most like attributed to tamoxifen's hepatotoxic effect. To know if Raman signature can accurately predict if a given cell is affected by tamoxifen or not. The data from 2 experiments were

used as a training data set (n = 290 cells) in a PLS-DA model. The predictive ability of this model was then tested against the data obtained from the third experiment (n = 120 cells). Our model was able to correctly classify cells treated with tamoxifen with 100% sensitivity, and 72% specificity. This shows that our experimental platform could accurately predict if cells are affected by tamoxifen or not, even in new data obtained from separate experiments. From this PLS-DA model, 13 peaks were isolated by calculating their VIP scores and found to be significantly different in treated and untreated cells. These peaks are hypothesized to be directly correlated to the drug-response on the single-cell level, therefore, they can be used as spectral biomarkers for the drug efficacy in future studies.

From the cells measured by Raman, 53 were picked up and measured by single-cell MS (31 treated cells, and 22 untreated cells). Our MS method could successfully detect both the drug, and its metabolite (4-HT) in single-cells treated with the drugs, the drug and its metabolite peaks were not present in the control cells. The structures of tamoxifen and 4-HT were confirmed by MS/MS analysis, therefore, showcasing the ability of our technique to monitor the drug and its metabolite on the single-cell level in a highly selective and sensitive manner. Furthermore, single-cell MS analysis revealed strong variation in tamoxifen and 4-HT concentrations across single-cells, where they exhibited %RSD of 151% and 238% respectively. The distribution of tamoxifen metabolism was also studied by calculating the ratio of metabolized to unmetabolized tamoxifen where it exhibited 222% RSD in its distribution. This perceived heterogeneity in the drug concentration could be explained by variations in the drug-uptake, while 4-HT abundance variations could be explained by variations in the uptake of its parent molecule, tamoxifen, as well as variations in its metabolism.

In order to explore the potential relationship between the drug or its metabolite concentration in single-cells and the drug-response, I studied possible correlations between the Raman peaks obtained from the PLS-DA model which might represent the drug effect, and the concentration of tamoxifen and 4-HT in single-cells. Among the 13 peaks chosen from the PLS-DA model, 6 correlated to either the drug, or its metabolite, or the ratio between the drug and its metabolite ($p < 0.05$). Furthermore, the ratios between 20 Raman peak pairs were found to be correlated with tamoxifen metabolism. These correlated peaks correspond to fatty acids, lipids, and proteins. This corroborates with recent literature about tamoxifen and 4-HT role in fatty acid oxidation, lipid metabolism, and membrane dynamics.

In light of our results, the Raman-MS integration was capable of identifying the response of single-cells to tamoxifen as well as quantifying the drug and its metabolite in the same cells, therefore, achieving pharmacodynamics and pharmacokinetics monitoring on the single-cell level for the first time.