A Continuously Infused Microfluidic Radioassay System for the Characterization of Cellular Pharmacokinetics

Zhen Liu¹#, Ziying Jian¹#, Qian Wang¹, Tao Cheng¹, Benedikt Feuerecker¹, Markus Schwaiger¹, Sung-Cheng Huang², Sibylle I. Ziegler¹, Kuangyu Shi¹

1. Dept. Nuclear Medicine, Technische Universität München, Munich, Germany
2. Dept. Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles, USA
# Contribute equally to the article

Running Title: Real-time Microfluidic Radioassay

Corresponding Author:
Zhen Liu
Dept. Nuclear Medicine,
Klinikum rechts der Isar
Technische Universität München
Ismaningerstrasse. 22
81675 Munich
Germany
Tel.: +49-89-4140 6033
Fax: +49-89-4140 7713
Email: liu@lrz.tum.de
**ABSTRACT**

Measurement of cellular tracer uptake is widely applied to understand the physiological status of cells and their interactions with imaging agents and pharmaceuticals. In-culture measurements have the advantage of less stress to cells. However, the tracer solution still needs to be loaded, unloaded and purged from the cell culture during the measurements. Here, we propose a continuously infused microfluidic radioassay (CIMR) system for continuous in-culture measurement of cellular uptake. The system was tested to investigate the influence of glucose concentration in cell culture medium on the 2-deoxy-2-(\(^{18}\text{F}\))fluoro-D-glucose (\(^{18}\text{F}-\text{FDG}\)) uptake kinetics.

**Methods:** The CIMR system consists of a microfluidic slide integrated with a Timepix positron camera. Medium diluted with radioactive tracer flows through the cell chamber continuously with a constantly low speed. Positrons emitted from the cells and from tracer in the medium are measured with the positron camera. Human cell lines SkBr3 and Capan-1 were incubated with medium of 3 different glucose concentrations and then measured with \(^{18}\text{F}-\text{FDG}\) on the CIMR system. In addition a conventional uptake experiment was performed. The relative uptake ratios between different medium conditions were compared. A cellular two compartment model was applied to estimate the cellular pharmacokinetics on CIMR data. The estimated pharmacokinetic parameters were compared with expressions of glucose transporter-1 (GLUT1) and Hexokinase-II (HK2) measured by quantitative real-time polymerase chain reaction (qPCR).

**Results:** The relative uptake ratios obtained from CIMR measurements significantly correlate with those from the conventional uptake experiments. The relative standard deviations of relative uptake ratios of CIMR are significantly lower than the conventional uptake experiments. The cellular two compartment model can fit with the \(^{18}\text{F}-\text{FDG}\) CIMR measurements with high fitting qualities. For SkBr3, the estimated pharmacokinetic parameters \(k_1\) and \(k_3\) are consistent with the mRNA expressions of GLUT1 and HK2, where the cultured conditions with low glucose concentrations lead to higher GLUT1 and HK2 expressions as well as higher estimated \(k_1\) and \(k_3\). For Capan-1, the estimated \(k_1\) and \(k_3\)
increase as the glucose concentration in culture medium increases, which does not match the corresponding mRNA expressions.

**Conclusion:** The CIMR system captures the dynamic cellular uptake within the cell culture. It enables the estimation of the cellular pharmacokinetics.

**Key Words:** Microfluidic radioassay; Positron imaging; Cellular pharmacokinetics; Glycolysis
INTRODUCTION

The investigation of molecular uptake inside cells is valuable for the understanding of physiological status (1,2), for the interpretation of molecular imaging (3,4), and for the development of imaging agents and pharmaceuticals (5). The cellular uptake of a molecule is usually assessed by measuring labelling signals such as radioactivity (2,6), optical signal (7) or magnetic signal (8). The radiometric measurement of cellular uptake is a typical experiment in nuclear medicine (6) and it provides high sensitivity for a very small mass amount of tracer. The assay offers the opportunity to study tracer behavior before translation to clinical applications. However, this method requires the separation of cells from the incubation medium to measure the radioactivity in the cells only. During this process, cells are processed and removed from the culture environment. Thus, only one single radioassay for a given cell culture sample can be performed (6). A high-resolution radioluminescence microscope was developed by Pratx et al. (2,9). It allows the measurement of cellular uptake of single cell by culturing cells in a thin scintillator and imaging using an invert microscopy with an electron-multiplying CCD camera (9). In another approach, the automatic measurement of real-time tracer binding was achieved on a radioimmunoassay system using a rotating cell dish (10). Vu et al. developed a microfluidic radioassay system (Betabox), which integrated a microfluidic chip with a position-sensitive β-camera (11). This system allows multiple radioassays of a small cell population directly in a culture environment. The cell cultures can be maintained for long time periods and the changes of the environment can be digitally controlled, which is very convenient for the investigation of diagnostic and therapeutic radiopharmaceuticals. The Betabox radioassay system has been utilized as a rapid screening platform for the investigation of the response of various cell lines to mechanistically distinct, targeted drugs (12,13). A cellular two-compartment model was developed to quantify the cellular pharmacokinetics based on the discrete measurements on BetaBox system (14).

Although the advantages of this pioneering microfluidic radioassay system are promising, it needs loading and unloading of the incubating tracers for each assay. Thus, the
measurements are restricted to several discontinuous time points and dynamic acquisition over a time course is still not possible. In addition, the medium exchange procedure needs to compromise between the completeness of the exchange medium and the maintenance of a stable culture environment (11,12). The application of this technique on less adhesive cell lines is therefore limited. Also, the residual of tracer medium in the cell chamber is difficult to control.

In this study, we developed a continuously infused microfluidic radioassay (CIMR) system to measure the dynamic information of cellular uptake and to improve the quantification of cellular tracer kinetics.

18F-FDG PET is widely used for in vivo tumor imaging based on the increased glycolysis in tumor cells known as Warburg effect (15-17). The uptake of 18F-FDG is considered as a biomarker of tumor malignancy and of prognostic value for therapy management. After entering into cells via glucose transporters (GLUTs), 18F-FDG is phosphorylated by Hexokinase (HKs) and gets trapped in the cells. When investigating the mechanism of 18F-FDG for tumor diagnosis and therapy response, the uptake of 18F-FDG is usually interpreted with regard to the expression of GLUTs and HKs of the pathway. Among the two families of proteins, GLUT1 and HK2 are mostly investigated. GLUT1 expression is usually considered to be associated with malignant tumor stages (18). 18F-FDG uptake was shown to be more influenced by GLUT1 than other subtypes of GLUTs in many tumor tissues such as breast cancer (19), pancreatic tumor (20) and cervical cancer (21). HK2 directly mediates glycolysis and promotes tumor growth (22) and it has been found to be the predominant isoform of HKs in many tumors (23,24). The overexpression of HK2 has been reported to increase 18F-FDG uptake in cancer cells (25). GLUT1 and HK2 have been employed as therapeutic target for drug development associated with cancer metabolism (26). On the other side, the expression of GLUT1 and HK2 varies among different types of cancer cells (27) and tumors possess special abilities to adjust the expression of GLUT1 or HK2 to maintain their energy supply and homeostasis (25). However, it is very difficult to investigate the adaption of their functions under various metabolic conditions (28,29).
As a proof of concept of the proposed CIMR system, we focused on the investigation of $^{18}$F-FDG uptake in tumor cells. The applicability of the proposed CIMR system was verified by comparing the estimated $^{18}$F-FDG kinetics with literature data and the corresponding expressions of GLUT1 and HK2 measured with conventional qPCR.

MATERIALS AND METHODS

Continuously infused microfluidic radioassay (CIMR) system

The continuously infused microfluidic radioassay system is based on a microfluidic chip ($\mu$-Slide VI0.4, ibidi GmbH, Munich, DE), a flow control unit and a positron camera as shown in Fig. 1. The microfluidic chip consists of two parallel chambers ($17 \times 3.8 \times 0.4$ mm$^3$ each), each one with separate inlet and outlet. The two chambers are connected as shown in Fig.1b. One of them serves as a medium monitoring chamber and the other as a cell culture chamber. The flow control unit is a programmable syringe pump (Cavro® XLP 6000, Tecan Group Ltd. Männedorf, CH) with connecting flow tubes. The medium with radioactive tracer is driven by the pump from the medium reservoir via the medium monitoring chamber into the cell culture chamber (Fig. 1B). The medium monitoring chamber and the cell culture chamber were simultaneously measured by a positron camera during the measurements (Fig. 1C). The positron camera consists of a single-particle counting silicon pixel detector (300 $\mu$m thickness, Crypix, Crytur Ltd. Turnov, CZ) bonded to a CMOS readout chip (Timepix, CERN, CH). The field of view of the positron camera is $14 \times 14$ mm$^2$ ($256 \times 256$ pixels). Details of the basic performance of this detector for direct positron measurement can be found in (30).

Cell experiments

Cell culture preparation. Two types of human cancer cell lines, Capan-1 (pancreas adenocarcinoma) and SkBr3 (breast adenocarcinoma), were selected for the investigation. Two days before the CIMR measurements, approximately $1.5 \times 10^4$ cells in 30 $\mu$l single-cell suspension were inoculated into the cell culture chamber of a microfluidic chip using a
Cells formed a homogeneous single layer on the bottom surface of the chamber. The cells were then cultured in 25 mM glucose DMEM medium (Biochrom GmbH, Berlin, DE), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at controlled condition of 37 °C and 5% CO₂. One day before the measurements, medium in the cell chamber was switched to special medium with 5mM, 2.5mM, or 0.5mM glucose concentration respectively. The corresponding medium was exchanged every 8 hours until the start of the measurements to sustain stable glucose concentration. At the time of the CIMR measurements, the cells reached approximately 60%-90% confluence inside the cell chamber.

**Measurements with the CIMR system.** Before the infusion, the flow control unit was sterilized and cleaned using 70% ethanol and ultrapure water. ¹⁸F-FDG was diluted into the cell culture medium to generate a radioactive solution of 0.2-5 MBq/mL with the investigated glucose concentration. The tracer medium solution was pumped through the medium chamber and cell chamber at a constant flow of 1.25 µl/s, which created a small shear stress (< 0.13 dyn/cm², estimated based on the application note of ibidi GmbH) near the chamber surface. This flow speed leads to a medium refresh rate of 2.5 times per minute, which is sufficient to maintain the continuous infusion profile. Positrons emitted both, from the medium chamber and the cell chamber, were measured by the positron camera for 65 min. Measurements were binned into frames of 1 min each. Each measurement was repeated 6 times.

After the CIMR measurements, the cell chamber was imaged using a microscope (BZ-9000, Keyence Co Ltd, JP) with a phase contrast objective lens of 20. Five widely distributed microscopic FOVs were selected for counting the number of cells. The average cell number of the 5 FOVs was then normalized to the cell number per detector pixel (55 × 55 µm²).

**Conventional uptake measurement**

As reference, FDG uptake in cells was studied with the conventional uptake method (6) for each of culture conditions used in the CIMR measurements. Two days before the uptake experiment, approximately 6 × 10⁴ cells in 300 µl suspension were inoculated into a well of a
24-well plate. One day before the uptake experiment, the cells were incubated in a medium of one of the three glucose concentrations used in the CIMR studies. Medium was refreshed every 8 hours before the uptake measurement. During the uptake measurement, $^{18}$F-FDG solution with the same glucose concentration used in the corresponding CIMR measurements was applied to the cells for 4 different time periods (10, 20, 30 and 40 min). Then the cells were washed twice with ice-cold PBS buffer and dissociated by trypsin/EDTA solution (Biochrom GmbH, Berlin, DE). The radioactivity of the collected cells in each well was measured using a gamma counter (Wallac 1470 Wizard Gamma-Counter, PerkinElmer, MA, USA). Thus, three repeated measurements of FDG uptake for incubation times of 10, 20, 30 and 40 min were obtained for each medium condition. The number of collected cells in each well was counted using the automated cell counter (Countess™, Invitrogen Life Technologies GmbH, Darmstadt, DE). The counted cell numbers of the wells with the same culture condition on the same day were averaged.

**qPCR.** Cells in the same conditions as in the CIMR measurements were cultured for molecular biology assays. Total RNA was extracted from each tumor cell line using the RNeasy Plus Mini Kit (QIAGEN, Limburg, NL); cDNA was prepared from the total RNA isolated by using QuantiTect Reverse Transcription Kit (QIAGEN, Limburg, NL). The sequences of primers for RT-PCR were as follows: GLUT1 (31): Forward: 5'-CAG GAG ATG AAG GAA GAG-3'. Reverse: 5'-TCG TGG AGT AAT AGA AGA C-3'. HK2 (22): Forward: 5'-CAA AGT GAC AGT GGG TGT GG-3'. Reverse: CAA AGT GAC AGT GGG TGT GG-3'. Housekeeping gene: Forward: 5'-CAG ATG GCA AGA CAG TAG AAG -3'. Reverse: 5'- GGC AAA AAT GGA AGC AAT GG-3'. All primers were synthesized by Eurofins Genomics. qPCR was performed on Roche Light Cycler480 Instrument I/II (Roche Applied Science, Penzberg, DE) with Light Cycler 480 SYBR Green I Master kit (Roche diagnostics). The data was analyzed by LightCyclerTM480 software version 1.05.0.39 (Roche diagnostics, Penzberg, DE).

**Data processing and analysis**
**Image processing and normalization.** The acquired dynamic data was corrected for radioactive decay. For each 1-minute frame, the mean events per pixel (55 × 55 µm²) within a box region of interest (12 × 3.8 mm²) in the center of an imaged chamber were calculated separately for the medium chamber and for the cell chamber. Time activity curves (TACs) were generated to describe the changes of mean events per pixel versus time. The calibrated radioactivity of the medium in the cell chamber was estimated based on the measured TAC of the medium chamber after correction of delay and dispersion. This can be described using the following formula (32):

$$\beta_{cm}(t) = \beta_m(t - \Delta T) \ast \frac{1}{\tau} e^{-\frac{t}{\tau}}$$

where $\beta_{cm}$ and $\beta_m$ denote, respectively, the event density curves of the medium in the cell chamber and the medium chamber, $\Delta T$ and $\tau$ are coefficients characterizing the delay and dispersion of the FDG activity flowing from the medium chamber to the cell chamber, and $\ast$ represents the convolution operator.

To estimate the delay and dispersion coefficients, the CIMR system was run 6 times without cells in the cell chamber using the same fluidic condition as the real measurements. The coefficients of the decay and dispersion parameters were determined regularly, especially after changes of the settings of the CIMR operating condition.

The microfluidic chamber has a height of 400 µm and the positron camera is placed below the chamber. Thus, the positron camera had lower sensitivity for the positrons emitted from upper layers than from bottom layers in the chamber. This depth-dependent sensitivity profile of the positron camera was corrected in the measurements by multiplying the event density by a depth-dependent sensitivity correction factor of 1.74 (see supplement 1).

The events of the cells in the cell chamber were generated by subtracting the estimated medium events in the cell chamber from the total events of the cell chamber (i.e., the events in the medium was removed from the total measured events in the cell chamber). The FDG concentration in the medium was computed by normalizing to the volume associated with a pixel in the chamber (55 × 55 × 400 µm). The cellular FDG concentration was calculated
using an estimated mean cell number and mean cell volume (see supplement 2). The mean volume of adherent cells with mean diameter \( d \) was approximated by the volume of an ellipsoid \( \pi d^3/12 \). The cellular uptake was estimated as the ratio of cellular FDG events (cps) per \( 10^6 \) cells over the corresponding medium FDG event density (cps/mL) (see supplement 2). The relative uptake ratios of CIMR were calculated as the ratios between the uptake TACs of the corresponding culture conditions for each time point. Similarly, the relative uptake ratios for conventional uptake experiment were computed as the ratios between uptake values at the discrete measurement times. In this study, the uptake ratios relative to that with the culture medium of 5 mM were calculated. All the processing was implemented using MATLAB 2012b (The Mathworks, Inc., Natick, MA, USA).

**Cellular pharmacokinetic modeling.** Based on the TACs obtained from the CIMR measurement, the pharmacokinetic parameters related to GLUT and HK can be estimated. For FDG, the uptake is controlled by the glucose transporter (GLUT) and hexokinase (HK) phosphorylation (supplement 3a). A cellular two compartment model was constructed to estimate the kinetic parameters relating to \( ^{18}\text{F}-\text{FDG} \) transport and phosphorylation (14) (supplement 3b). Given the medium event density \( \beta_{cm} \), the free FDG events in cell \( \beta_{in} \) and the phosphorylated FDG events \( \beta_{ph} \) in cell can be described as following

\[
\frac{d\beta_{in}(t)}{dt} = k_1\beta_{cm}(t) - k_2\beta_{in}(t) - k_3\beta_{in}(t) + k_4\beta_{ph}(t)
\]

\[
\frac{d\beta_{ph}(t)}{dt} = k_3\beta_{in}(t) - k_4\beta_{ph}(t)
\]

where \( k_1, k_2, k_3 \) and \( k_4 \) are rate constants. \( k_1 \) and \( k_2 \) stand for the import and export rates of FDG across the cell membrane respectively. \( k_3 \) and \( k_4 \) are the phosphorylation and de-phosphorylation rates of FDG inside cell. The parameters were estimated by fitting the TACs derived from CIMR measurements. The model fit was performed by a trust-region algorithm implemented using C++ with Intel MKL library.

A Michaelis-Menten equation was applied to interpret the relation between the estimated rate constants (\( k_1 \) and \( k_3 \)) and mRNA expressions of the corresponding enzyme or
transporter (33):

\[ k \sim \frac{V_{\text{max}}}{K_m + C_{\text{endo}}} \]

where \( C_{\text{endo}} \) is the concentration of the endogenous substrate, \( K_m \) is the substrate concentration at half maximum reaction rate, and \( V_{\text{max}} \) is the maximum reaction rate, which is assumed to be proportional to protein expression. Considering that medium glucose concentration was kept constant before and during CIMR, the endogenous glucose concentration was assumed to be the same as the medium glucose concentration. The FDG concentration is negligible compared to the medium glucose concentration. For each cell line, we used fitting of the Michaelis-Menten equation to understand if the \( K_m \) rate can be the same for culturing with different glucose concentrations. Nonlinear least square fit was applied to fit the cellular pharmacokinetic parameter with the mRNA expressions.

Pearson correlation was applied to compare the relative uptake ratios between CIMR and conventional uptake experiments. The paired Student’s t-test was used to further compare the relative standard deviation (std/mean) of the two methods. A significance level of \( p<0.05 \) was established.

RESULTS

During the continuous infusion procedure, 1.3-4.5% (3.2±1.7%, n=3) loss of cells were observed for Capan-1, and 0.9-3.5% (1.9±1.4%, n=3) loss of cells were observed for SkBr3. The difference in sensitivity of the medium chamber and the cell chamber with no cells ranged from 0.3 to 1.1% (0.6±0.3%, n=6).

The modeled curves including delay and dispersion could fit the measured curves well for the 6 calibration runs with no cells in the cell chamber (\( R^2=0.9995±0.0003 \)). One example is shown in Fig. 2A. The estimated delay \( \Delta \tau \) (2.11±0.11 min) and dispersion coefficient \( \tau \) (1.70±0.11 min\(^{-1}\)) have small relative standard deviation (<7%). When the mean delay and dispersion coefficients of the 6 curves were applied to predict the TAC in the cell chamber, the absolute percentage error was 1.3±0.3% for the 6 runs (Fig. 2A).
The measured data of CIMR were well fitted by the cellular two-compartment model with high fitting quality ($R^2>0.9999$), for both SkBr3 and Capan-1 under the investigated conditions. Fig. 2B shows an example of the model fitting for SkBr3 cultured using medium with glucose concentration of 0.5 mM. The predicted medium events in the cell chamber (after correction for delay and dispersion) are plotted as the red curve. After fitting using the cellular two compartment model, the modeled total event curve fitted well to the measured total events in the cell chamber.

Mean and standard deviation of the estimated cellular uptake (mL / $10^8$ cells) on CIMR system for 6 repeated measurements are plotted (in Fig. 3). For the same cell under the same condition, the uptake followed a similar trend. The averaged relative standard deviation of normalized uptake for SkBr3 in the 3 culture media was 18.5% (5 mM), 15.1% (2.5 mM) and 5.4% (0.5 mM) (Fig. 3a). For Capan-1, the corresponding averaged relative standard deviation was 8.8% (5 mM), 8.0% (2.5 mM) and 9.1% (0.5 mM) (Fig. 3B).

The relative uptake ratios of CIMR and conventional uptake experiments for the two investigated tumor cell lines are shown (in Fig. 4). The mean and standard deviation are plotted. Significant correlation has been found between CIMR and conventional uptake experiments for either SkBr3 ($r=0.98$, $p=9\times10^{-6}$) as well as Capan-1 ($r=0.95$, $p=0.0003$). Overall, the CIMR methods have 11.3% relative standard deviation, which is significantly less ($p=0.004$) than the uptake experiments of 20.8%.

The $k_1$ of the SkBr3 cells cultured with 0.5 mM glucose concentration is approximately 75% higher than with glucose concentration of 2.5 mM and 5 mM (Fig. 5). This is in agreement with the qPCR results of GLUT1 mRNA level, where the GLUT1 expression of cells with medium of 0.5 mM glucose is around 60% higher than for 2.5 mM and 5 mM medium concentrations. Similarly, the $k_3$ of the cells cultured with glucose concentration of 0.5 mM is higher than in the case of 2.5 mM or 5 mM. The HK2 mRNA levels of cells with medium of glucose 0.5 mM is larger than 2.5 mM and 5 mM.

The $k_1$ of the Capan-1 cells cultured with glucose concentration of 0.5 mM is around 25% higher than with glucose concentration of 2.5 mM or 5 mM (Fig. 6). However, the qPCR
results of GLUT1 expression of cells with medium of glucose of 5 mM is higher than for 2.5 mM and 0.5 mM. The $k_3$ of the cells cultured with glucose concentration of 0.5 mM is more than 60% higher than 2.5 mM and 5 mM. However, only less than 25% increase of the HK2 mRNA levels of Capan-1 cells was observed when the condition of 0.5 mM glucose was compared with that of 2.5 mM and 5 mM glucose.

For SkBr3, the estimated cellular kinetics can be fitted with the mRNA expressions using a fixed $K_m$ with the determinant coefficient ($R^2=0.73$ for $k_1$ and $R^2=0.97$ for $k_3$). However, for Capan-1, it is not possible to fit the estimated cellular kinetics with the corresponding mRNA levels using a fixed $K_m$.

**DISCUSSION**

Similar to the previous approach BetaBox (discontinuous microfluidic radioassay) (11), the proposed continuously infused microfluidic radioassay (CIMR) allows in-culture measurements. It does not require loading, unloading, and cleaning of the tracer medium in the culture chamber. Thus, the measurements can deliver direct cellular uptake information without introducing additional stress during the medium exchange process. In particular, the continuous measurement captures the full dynamic course of cellular uptake. This enables the application of pharmacokinetic analysis. The results of these initial tests showed that this system can achieve reproducible cellular uptake measurements as well as stable estimation of cellular kinetics. However, the volume of the chamber of the microfluidic chip in our system is much larger than that in the Betabox system. This is necessary to get sufficient cell uptake signals to distinguish the background medium events during the mixture measurements of cell uptake and medium.

The medium chamber in the CIMR system monitors the medium events in the cell chamber. However, the measured medium events need to be corrected for delay and dispersion before the TAC can be used to correct the measurement of the cell chamber. The effective volume of the microfluidic chamber is 30 µL, the volume of two connectors is 56.5 µL each, and the volume of the tubing in between is 14.1 µL. Under the experimental flow
speed, the theoretical delay between cell chamber and medium chamber is 2.09 min. The estimated delay coefficient $\Delta T = 2.11$ min agrees with the theoretical estimation. Although the volume between the two chambers may slightly vary due to the variability by connecting the chambers with the tubes manually, the small relative standard deviation has little influence (<2%) on the predicted medium events of the cell chamber and can be ignored for high uptake signals.

The CIMR system is operating in a continuous infusion condition and the fluid shear stress on cells is less than 0.13 dyn/cm$^2$. In the physiological condition, the interstitial flow shear stress on normal tissues has been demonstrated to be in the order of 0.1 dyn/cm$^2$ or lower (34). Thus, the fluid shear stress in our system is similar to that in the physiological conditions. In addition, the low cell loss (<4.5%) during the CIMR measurements shows that this low shear stress does not show significant influence on the adherence of the two investigated cells in the chamber. However, for less adherent cells, even low shear stress may lead to non-negligible cell loss. Further strategies need to be developed to compensate the influence of leached-out cells for those less adherent cells. Otherwise, such cells are not suitable for the CIMR measurements.

The uptake values obtained in CIMR are calculated from event densities of medium chamber and cell chamber, which are not absolute activity concentrations. The sensitivity of the system (calibration of event density to absolute activity concentration) is not considered during the calculation. In this system, we put a mylar sheet (6 µm) between the microfluidic chip and the detector to prevent possible leakage of fluid onto the semiconductor detector during port exchange. The air space between the microfluidic chip and the detector may change for different set ups, leading to slightly varying absolute sensitivity. However, in this study, we measured the medium chamber and the cell chamber simultaneously. Thus, the sensitivity change did not affect the calculation of the uptake, nor the estimation of kinetic parameters (proof in supplement 4). The difference of absolute sensitivity observed between the medium chamber and the cell chamber was less than 1.1% (0.6±0.3%), thus the same sensitivity was assumed for both chambers. Even after careful calibration and correction, the
estimated uptake values were observed to be different from the conventional uptake values obtained from well counter measurements (supplement 5). This may be explained by the difference between in-culture measurement and conventional ex-culture measurements. The influences of sample preparation procedure (wash with cold PBS, dissociate attached cells with trypsin) on cellular uptake are not known and further investigations are necessary to understand the difference between in-culture measurement and conventional uptake measurements. Nevertheless, the significant correlations between the relative uptake ratios (0.5 mM / 5 mM, 2.5 mM / 5 mM) of the two types of measurements demonstrated the consistency of relative relations between each other. As many studies investigate the relative differences under certain interventions, the systematic bias between CIMR and conventional uptake may not change the results if all the investigations were performed in in-culture measurements (11). Smaller variations were achieved with the in-culture measurement using CIMR, compared with the conventional uptake experiments. Thus, the CIMR provides a stable way to investigate relative changes under interventions.

Furthermore, the uptake values obtained in CIMR are indirect estimations compared with the previous in-culture microfluidic radioassay using a loading and flushing protocol (11). By adapting the infusion profile of CIMR with flushing the tracer, the pure event density of cells can be measured and the calculated uptake values are consistent with the continuously measured CIMR uptake (supplement 5). This supports the feasibility of the continuous estimation of uptake during the infusion without flushing the tracer.

For the estimation of cellular pharmacokinetics, it is necessary to estimate the concentration (event density) inside and outside of the cells. For the event density inside cells, the cell volume needs to be considered. However, the real cell volume is difficult to measure and was therefore estimated by the diameters of cells in the cell chamber. This may introduce bias for the parameter estimation. Nevertheless, the estimated kinetic parameters are consistent with the data of the same cancer in literature. For glucose concentration of 5 mM (corresponding to the human condition), the fitted phosphorylation rates ($k_3$) of this study ranged from 0.027 to 0.054 min$^{-1}$ for the breast cancer cell line SkBr3.
This is in agreement with the reported parameter range of 0.025 to 0.061 min\(^{-1}\) (35) and 0.012 to 0.078 min\(^{-1}\) (36) for dynamic \(^{18}\)F-FDG PET on human breast cancer in literature. Similarly, the fitted \(k_3\) of this study ranged from 0.031 to 0.055 min\(^{-1}\) for pancreatic cancer cell line Capan-1 for glucose concentration of 5 mM. This is also in line with a reported \(k_3\) value 0.041 min\(^{-1}\) (separating the overall survival between 4 and 6 months) for \(^{18}\)F-FDG PET on human pancreatic adenocarcinoma patients (37).

In the current set-up, the infusion protocol (input function) is a step function, which results in short transient response followed by a linear TAC. This may introduce bias in the estimation. Further constraints can be added by adapting the infusion protocol, such as a square pulse function. In supplement 6, two different square function infusion profiles are tested for the estimation of the kinetic parameters on SkBr3 cells cultured using 0.5 mM glucose. Comparing the estimations using square function and step function, the values of \(k_1\) from the square function (1.17±0.27) are slightly higher than that (0.99±0.26) of the previous step function. But no significant difference has been observed using non-paired t-test. The values of \(k_3\) of the square function (0.13±0.08) are almost the same as that (0.14±0.04) of the step function, no significance was observed. The variation with the square function is slightly larger than the step function. There might be bias in the estimation using different infusion profiles (input functions). For the studies in the manuscript, all the \(k_4\) values are nearly 0. Although the square infusion profile may improve the estimation and reduce the bias compared with the step infusion profile, it does not bring significant difference for the studies without obvious dephosphorylation. However, the square function may bring significant improvement for the investigation of tracers with clear \(k_4\) clearances.

The estimated kinetic parameters of this study cannot directly be linked to underlying physiological behavior. In this study, we employed a quantitative index, the mRNA level of corresponding proteins measured using qPCR, for comparison. However, the expression levels of the corresponding proteins may deviate from the mRNA expression levels (38). The investigated typical glucose transporter GLUT1 and typical phosphorylation enzyme HK2 may not represent the overall function of multiple existing isoforms of GLUT and HK. In
addition to protein expressions, the activities of the transporters and enzymes also influence the behavior of the transport and phosphorylation (28). In contrast, the estimated kinetic parameters represent the overall effects of protein expression and activity. Furthermore, it is not always possible to fit the pharmacokinetic parameters with mRNA expressions using the Michaelis-Menten equation (33) with a constant $K_m$ (failed for Capan-1). For each cell line, we tried to estimate $K_m$ for various glucose incubation conditions. We used the model fitting to test if it is possible to interpret the relation between the mRNA expression and kinetic parameters using a constant $K_m$. A change in the value of the rate constant is not expected to match the change in $V_{max}$, especially, when the concentration of the substrate (glucose level in the present case) was altered, unless the value of $K_m$ is much larger than the substrate concentration. Thus, it is not expected that the mRNA levels agree with the cellular pharmacokinetics of $^{18}$F-FDG in all the situations.

All the current CIMR measurements were performed under the condition of normal air, room temperature (ca. 25 Cº) and room humidity, without the use of a dedicated incubator. Although we tried to minimize the measurement time outside of the incubator, this may still introduce stress to cells during the measurements leading to bias of the estimation of cellular uptake. Nevertheless, all the environmental conditions were similar for the various runs performed in this study. Their variations were small compared to the variabilities due to the many other factors addressed earlier.

The CIMR system can be extended to measure positrons or electrons emitted by other tracers using the same detector in this study (39,40). The measured signal depends of the energy of the emitted positron or electrons. For different tracers with the same positron emitter, i.e. $^{18}$F labeled tracer, the proposed methods can be directly transferred. For tracers with different positron emitters like $^{68}$Ga, $^{11}$C, some correction coefficients, such as decay correction, or depth-dependent sensitivity correction coefficient need to be recalculated.

CONCLUSION

This study developed a continuously infused microfluidic radioassay system for real-time
in-culture measurement of cellular uptake of a radiotracer. It allows the estimation of cellular pharmacokinetics based on the dynamic measurements of the uptake time course of the radiotracer. The initial experiments demonstrated the reproducibility, stability and capability of capturing pharmacokinetic differences of the system. It provides a platform for convenient quantitative investigation of cellular physiology and pharmacokinetics.

ACKNOWLEDGMENTS

The research leading to these results is based on the funding from German Research Foundation (DFG) Collaborative Research Centre 824 (SFB824). It has also been supported by China Scholarship Council. The scientific collaboration between UCLA and TUM was supported by BaCaTec (Project 17, 2012-1). The authors thank Birgit Blechert, Dr. Christine Bayer, Dr. Daniela Schilling for providing cell lines, Prof. Markus Essler for valuable discussions, Dr. Jan Tous (CRYTUR, spol. s r.o.) for support related to the positron camera and Dr. Armin Bieser (iBidi GmbH) for help on the microfluidic chips.

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**Fig. 1.** Setup of the continuously infused microfluidic radioassay (CIMR) system: (A) a photo of the microfluidic chip in operation; (B) a sketch of the tracer medium flow during the measurement; (C) a side view at the line of view cut on panel b for the cell chamber during the measurement; (D) an example frame from the positron camera during the CIMR measurement.
Fig. 2: Illustration of model fitting: (A) model fitting for delay and dispersion and the predicted curve using the fit results; (B) pharmacokinetic model fitting for cellular uptake of SkBr3 cells cultured with a medium of 0.5 mM glucose.
**Fig. 3:** Plots of the mean and std of the normalized uptake curves for 6 repeated measurements on CIMR with 3 different culturing conditions for: (A) the cell line SkBr3; (B) the cell line Capan-1.

**Fig. 4:** Comparison of relative uptake ratio between CIMR uptake TACs and conventional uptake measurements of (A) SkBr3 and (B) Capan-1. The mean and standard deviation of 6 repeated measurements of each type were plotted.
Fig. 5: Comparison of cellular pharmacokinetics of CIMR and qPCR for SkBr3 with culture medium of different glucose concentration (5 mM, 2.5 mM & 0.5 mM): (A & B) plots of the estimated cellular pharmacokinetics $k_1$ and $k_3$ based on CIMR data; (C & D) plots of qPCR measurements of GLUT1 and HK2 for cells with the same culture conditions.
Fig. 6: Comparison of cellular pharmacokinetics of CIMR and qPCR for Capan-1 with culture medium of different glucose concentration (5 mM, 2.5 mM & 0.5 mM): (A & B) plots of the estimated cellular pharmacokinetics $k_1$ and $k_3$ based on CIMR data; (C & D) plots of qPCR measurements of GLUT1 and HK2 for cells with the same culture conditions.
**Supplement 1: Depth-dependent sensitivity correction**

The sensitivity of the positron detector decreases as the distance between the detector and the layer of the medium increases because positrons interact before they reach the detector. The depth-dependent sensitivity profile of the Timepix chip was calibrated by a depth-dependent correction factor, which is estimated based on the Monte-Carlo simulations using Geant4, a toolkit modeling the physical processes of particles passing through matter (1).

The simulation of the positron measurement using the Timepix chip was verified in previous studies (2, 3). As the above figure displays, a surface source emitting $^{18}$F positrons arbitrarily from a box region ($5 \times 5$ cm$^2$) was placed on top of a plastic layer of thickness 180 µm, a mylar layer of 6 µm and an air layer of 20 µm. The energy of the emitted positrons followed the theoretical $^{18}$F positron energy spectrum (maximum energy $E_{\text{max}} = 633$ keV). Then layers of water with thickness of 20 µm were inserted one after each other between the surface source and the plastic layer. The depth-dependent sensitivity profile was derived as:

$$f(x) = -0.00104x + 0.4732 \text{(cps/Bq)}$$

where $x$ denotes the thickness of water between the source and the plastic layer. In the microfluidic study, the medium is diluted with radioactivity. Given an investigated region of area $A$ and a radioactivity concentration of the medium of $\rho$, the counted events $y$ of the positron detector is a function of the thickness of the medium $l$,

$$y(l) = \int_0^l f(x)Ad\rho = -0.00052A\rho l^2 + 0.4732A\rho l$$

The correction factor $\alpha$ for a thicker layer (thickness $L_2$) to a thin layer (thickness $L_1$) is as following
\[
\alpha = \frac{L_2}{L_1} \left( \frac{\gamma(L_2)}{\gamma(L_1)} \right) = \frac{(-0.00052L_1^2 + 0.4732L_1)L_2}{(-0.00052L_2^2 + 0.4732L_2)L_1}
\]

In this study, the average diameter of the cell is 45.9 µm and we assume the thickness of the cell is 22.95 µm. Thus, the correction factor \( \alpha \) applied in this study is 1.74.

Supplement 2: Procedure of data processing

Supplemental Fig. 2
**Supplement 3**: Sketches of (A) the procedure of FDG uptake and (B) the corresponding cellular pharmacokinetic modeling.

**Supplemental Fig. 3**
Supplement 4: Influence of sensitivity

The processing of data in this study does not consider the absolute radioactivity concentration. Instead, it estimates the uptake values and the kinetic parameters using event density based on measurements of captured positron events in positron detector. Assuming the sensitivity of the detector to the cell layer is θ and the medium events has already been corrected for depth-dependent sensitivity to the cell layer (see supplement 1). For delay and dispersion correction, the measured medium event density in the cell chamber without cells is $\beta_{cm}(t)$ and the measured medium events in medium chamber is $\beta_{m}(t)$.

$$\beta_{cm}(t) = \beta_{m}(t - \Delta T) * \frac{1}{\tau} e^{-t/\tau}$$

The absolute activity concentration $C_{m}(t)$ in medium chamber is

$$C_{m}(t) = \frac{\beta_{m}(t)}{\theta}$$

The absolute activity concentration of medium in cell chamber $C_{cm}(t)$ is

$$C_{cm}(t) = \frac{\beta_{cm}(t)}{\theta} = \frac{\beta_{m}(t - \Delta T) * \frac{1}{\tau} e^{-t/\tau}}{\theta}$$

As θ is constant during the same measurement, we could derive

$$C_{cm}(t) = C_{m}(t - \Delta T) * \frac{1}{\tau} e^{-t/\tau}$$

Thus, the delay and dispersion correction are not influenced by the sensitivity and can be applied to event density.

Given the event density of cells in cell chamber $\beta_{cc}(t)$, the normalized uptake

$$\omega(t) = \frac{\beta_{cc}(t)}{\beta_{cm}(t)} = \frac{\beta_{cc}(t)/\theta}{\beta_{cm}(t)/\theta} = \frac{C_{cc}(t)}{C_{cm}(t)}$$

where $C_{cc}(t)$ is the absolute activity concentration of cells in cell chamber. It is equivalent to the normalized uptake using absolute activity concentration.

Further, for the cellular pharmacokinetic modeling, the modeling equation can be derived (4)

$$\beta_{cc}(t) = \beta_{in}(t) + \beta_{ph}(t) = a_1 e^{-b_1 t} * \beta_{cm}(t) + a_2 e^{-b_2 t} * \beta_{cm}(t)$$
where,

\[
\begin{align*}
    a_1 &= \frac{k_1(b_1 - k_3 - k_4)}{\Delta} \\
    a_2 &= \frac{k_1(b_2 - k_3 - k_4)}{-\Delta} \\
    b_1 &= \frac{k_2 + k_3 + k_4 + \Delta}{2} \\
    b_2 &= \frac{k_2 + k_3 + k_4 - \Delta}{2} \\
    \Delta &= \sqrt{(k_2 + k_3 + k_4)^2 - 4k_2k_4}
\end{align*}
\]

As the absolute activity concentration of cells in cell chamber \(C_{cc}(t)\)

\[
C_{cc}(t) = \frac{\beta_{cc}(t)}{\theta} = \frac{a_1 e^{-b_1t} \ast \beta_{cm}(t) + a_2 e^{-b_2t} \ast \beta_{cm}(t)}{\theta}
\]

As \(\theta\) is constant and

\[
C_{cm}(t) = \beta_{cm}(t) / \theta
\]

Thus, we can derive

\[
C_{cc}(t) = a_1 e^{-b_1t} \ast C_{cm}(t) + a_2 e^{-b_2t} \ast C_{cm}(t)
\]

Using the event density is equivalent to using absolute radioactivity concentration in the parameter estimation of cellular pharmacokinetic modeling.

**Supplement 5:** Comparison of uptake obtained on CIMR and conventional uptake experiment using well counter: A) compare the estimated uptake between CIMR after flushing the tracer out at 30 and 40 min and conventional ex-culture uptake (n=3); B) compare the relative uptake of A by normalizing each uptake to the mean values to 1; C) compare two CIMR uptakes, 1) the estimated real-time uptake values using continuous infusion profile, 2) uptake values after flushing the tracer out at 30 and 40 min.

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**Supplemental Fig. 4**
**Supplement 6**: A test of CIMR using square function infusion profile:  
A) an example measurement with flushing the tracer out at 30 min post infusion and the corresponding curve fitting;  
B) an example measurement with flushing the tracer out at 40 min post infusion and the corresponding curve fitting;  
C) compare the k1 parameters using step function (NoFlush, n=6), flushing at 30 min (Flush30, n=3), flushing at 40 min (Flush40, n=3) and the summary of Flush30 and Flush40 (Flush, n=6);  
D) compare the k3 parameters using step function (NoFlush, n=6), flushing at 30 min (Flush30, n=3), flushing at 40 min (Flush40, n=3) and the summary of Flush30 and Flush40 (Flush, n=6);

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**Supplemental Fig. 5**