MASTERARBEIT | MASTER'S THESIS

Titel | Title

Key Factors impacting the measurement of [^{18}F]FDG cell uptake

verfasst von | submitted by

Ilva Kristiana Langrate

angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien | Vienna, 2024

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 066 606
Studienrichtung lt. Studienblatt | Degree programme as it appears on the student record sheet:	Masterstudium Drug Discovery and Development
Betreut von | Supervisor:	Mag. Dr. Cecile Philippe

Kurzfassung

Die genaue Messung der Aufnahme von Arzneimitteln in Zellen ist wichtig für das Verständnis der Wirksamkeit von Medikamenten. Die Wahl der Versuchsparameter, einschließlich des Plattenmaterials, die Dauer der Inkubationszeit, die Zusammensetzung der Zellmedien und die Art des Experiments, kann auf den ersten Blick unspezifisch erscheinen. Diese scheinbar unbedeutenden Details können jedoch einen tiefgreifenden Einfluss auf ein ansonsten erfolgreiches Experiment haben und zu falsch positiven oder negativen Ergebnissen führen.

In dieser Untersuchung wird ein umfassender Ansatz zur Bewertung der [^{18}F]FDG-Aufnahme in CHO- und PC-3-Zellen durch Optimierung der verschiedenen Versuchsbedingungen. Die Methoden umfassen den Vergleich der Ergebnisse der zellulären [^{18}F]FDG Aufnahme bei Verwendung hydrophiler PVDF und Glasfaserplatten, die Bewertung der Aufnahme in membrangebundenen und freien Zellen, die Bewertung der Auswirkungen auf die zelluläre Aufnahme bei Verwendung von Medien mit und ohne Glukose, Prüfung Einfluss unterschiedlicher Inkubationszeiten (30 min, 60 min, 90 min) auf die Aufnahmeeffizienz.

Diese vergleichende Analyse zeigt, wie sich experimentelle Variablen auf die zelluläre Aufnahmemessungen auswirken. Hydrophile PVDF-Membranplatten zeigen überragende Wirksamkeit bei der Arzneimittelaufnahme, während eine längere Inkubationszeit (90 min) die [^{18}F]FDG-Aufnahme sowohl in CHO- als auch in PC-3-Zellen. Außerdem erhöht die Abwesenheit von Glukose in den Medien die Aufnahme des Medikaments, insbesondere in PC-3-Zellen. Bei der Auswahl einer zellulären Aufnahmemethode zeigt die membranbasierte Methode einen statistisch signifikanten Unterschied im Vergleich zur konventionellen Methode.

Schlüsselwörter: [^{18}F]FDG, CHO-Zellen, PC-3-Zellen, zelluläre Aufnahme, experimentelle Faktoren.

Abstract

Accurately measured drug uptake within cells is important for understanding drug efficacy. The choice of experimental parameters, including the plate material, duration of incubation period, composition of cell media and type of experiment, may appear unspecific at first. However, these seemingly minor details can exert a profound influence on an otherwise succesful experiment, leading to false positive or negative outcomes.

This study presents a comprehensive approach to evaluate [^{18}F]FDG uptake in CHO and PC-3 cells by optimizing different experimental conditions. The methods include comparing results of cellular [^{18}F]FDG uptake when using hydrophilic PVDF and glass fiber plates, assessing uptake in membrane-bound and free cells, evaluating effects on cellular uptake when using media with and without glucose, testing influence of different incubation time (30 min, 60 min, 90 min) on uptake efficiency.

This comparative analysis highlights how experimental variables impact cellular uptake measurements. Hydrophilic PVDF membrane plates demonstrate superior efficacy in drug uptake, while prolonged incubation period (90 min) enhance [^{18}F]FDG uptake in both CHO and PC-3 cells. Moreover, the absence of glucose in the media increases drug uptake, particularly in PC-3 cells. When selecting a cellular uptake measurement technique, the membrane-based method demonstrates a statistically significant difference compared to the conventional method.

Keywords: [^{18}F]FDG, CHO cells, PC-3 cells, cellular uptake, experimental factors.

Acknowledgements

“Tu tikai turi acis ciet,

Lai laime nepāriet,

Lai laika nepietiek,

Lai, lai, lai, lai...”

Ģirts Lūsis

Vismīļākais PALDIES māmiņai, tētim, memmei, māsiņai Anniņai, Reinim.

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Abbreviations.

1. **ATP -** adenosine triphosphate
2. **CHO cells –** Chinese hamster ovary cells
3. **DMEM -** Dulbecco's Modified Eagle Media
4. **[^{18}F]FDG –** 2-deoxy-2-[^{18}F]fluoro-D-glucose
5. **FBS –** fetal bovine serum
6. **HEPES -** (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
7. **IP –** incubation period
8. **NADPH -** nicotinamide adenine dinucleotide phosphate
9. **PABA -** para-aminobenzoic acid
10. **PBS** - phosphate buffered saline
11. **PET/CT -** positron emission tomography/computed tomography
12. **PC-3 cells -** human prostate cancer cell line
13. **PVDF -** polyvinylidene fluoride

Introduction.

Drug discovery and development process involves several pivotal stages, starting from adressing the problem, target identification, hit-to-lead optimization, and numerous preclinical experiments both in vitro and vivo.[1] Despite meticulous execution and accuracy in each step, use of incorrect equipment or methodology to interpret results may lead to mismatch with the actual reality. This underscores a critical need for vigilance in selecting and employing precise tools and techniques to ensure the reliability and validity of the entire drug development process.

In the realm of drug development and pharmacology, it is essential to employ diverse measurement methodologies to validate and cross-reference results, ensuring a robust assessment of drug uptake across various contexts. It also emphasizes the need for standardized protocols, promoting consistency across diverse experimental settings. Techniques such as membrane-based method and conventional cellular uptake measurement technique provide valuable insights into the drug uptake within cells. However, each method has its limitations and biases, underscoring the importance of employing a multimodal approach to enhance the reliability and accuracy of the data obtained.

Exploring different materials for culture plates, for instance, can impact cell adhesion, proliferation, and overall behavior. This, in turn, may influence the accessibility of cells to the administered drug and alter uptake patterns. Hydrophilic PVDF membrane plates are frequently employed in chromatography and other various

[1] Hughes, J. P., Rees, S., Kalindjian, S. B., & Philpott, K. L. (2011). Principles of early drug discovery. British Journal of Pharmacology, 162(6), 1239-1249. https://doi.org/10.1111/j.1476-5381.2010.01127.x

instrumental analyses due to their minimal protein binding, enhanced flow rate, and superior chemical resistance and thermal stability.[2] Conversely, glass fiber filter membrane plates possess outstanding wet strength, making them particularly well-suited for qualitative analyses and prefiltration of heavily contaminated samples.[3] The choice between glass-fiber or hydrophilic membrane plates depends on the specific requirements of the application and the characteristics of the samples being processed. By employing these diverse plates and utilizing a gamma counter for drug uptake measurement, we aim to discern any variations in drug uptake measurements resulting from the selection of plate material.

Variations in the incubation period provide insights into the kinetics of drug internalization, shedding light on the optimal duration for observing changes in cellular uptake without compromising cell viability. Cellular drug uptake can be affected by different incubation periods as the length of the incubation period influences the detectable signal intensity. An extended incubation period may introduce cytotoxic effects due to the reagents which utilize cellular energy, causing biological and functional impairements of the cells, higher cell death level and higher risk of cell adherence to the walls. Based on Betzer et al., the capacity of gold nanoparticle uptake

[2] Membrane Solutions. Hydrophilic PVDF Membrane. Retrieved from https://www.membrane-solutions.com/pvdf_hydrophilic_membrane.htm

[3] Merck Millipore. (n.d.). MultiScreen Filter Plates with Glass Fiber Filters. Retrieved from https://www.merckmillipore.com/AT/de/product/MultiScreen-Filter-Plates-with-Glass-Fiber-Filters,MM_NF-C76299?ReferrerURL=https%3A%2F%2Fwww.google.com%2F

stabilizes within one hour of incubation in 37°C, but incubation period of 90 min can cause impairment of immune cell activity.[4,5]

The composition of cell media, including the presence of specific nutrients, growth factors, or serum components, can modulate cell physiology and, consequently, impact drug uptake. The interplay between these factors and the drug under investigation should be carefully considered to draw accurate conclusions about its efficacy in different cellular environments.

Glucose is the most commonly added sugar in various cell medias, acting as the main energy source for cells and is necessary for both healthy and diseased cells to proliferate and maintain homeostasis.[6,8] Through anaerobic glycolysis, a single glucose molecule yields two ATP molecules, while the resulting pyruvate becomes a substrate for the mitochondrial tricarboxylic acid cycle, contributing to oxidative phosphorylation. Consequently, it is widely acknowledged that media for various cell cultures necessitate elevated glucose concentrations to enhance cell proliferation. Highly proliferative cells, including cancer cells, exhibit a preference for glucose as their primary fuel source to sustain rapid growth.[7] When dealing with glucose-dependent healthy or diseased cells, the presence of excess glucose in the media could

[4] Weifeng, L., Zhenlin, F., Yan, L., & Tian-Yun, W. (2021). Serum-free medium for recombinant protein expression in Chinese hamster ovary cells. Frontiers in Bioengineering and Biotechnology, 9. https://doi.org/10.3389/fbioe.2021.646363

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[6] Riss, T. L., Moravec, R. A., Niles, A. L., et al. (2013, May 1). Cell Viability Assays. In S. Markossian, A. Grossman, M. Arkin, et al. (Eds.), Assay Guidance Manual. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences. 2004-. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK144065/

[7] Jang, M., Kim, S., & Lee, J. (2013). Cancer cell metabolism: Implications for therapeutic targets. Experimental & Molecular Medicine, 45(e45). https://doi.org/10.1038/emm.2013.85

potentially interfere the uptaking of glucose-bound radiopharmaceuticals during incubation period. This raises an important question: does the supplementary glucose in the media have a detrimental impact on the uptake of these glucose- based drugs or radiotracers on healthy and diseased cells.

The physiological state of the cells under investigation plays an important role in influencing drug or radiotracer uptake. Attention must be given to optimizing experimental conditions to mimic the physiological environment as closely as possible. Variations in cell membrane permeability, endocytic pathways, intracellular trafficking mechanisms and overall condition of cells can significantly impact drug uptake kinetics. To comprehensively explore this phenomena, healthy CHO and pathological PC-3 cell lines were used.

CHO cells are one of the most commonly used mammalian cells in medical and pharmaceutical research and are utilized in various applications, such as recombinant protein production including therapeutic monoclonal antibodies, vaccine development, gene therapy research, and novel drug screening.[8,9] CHO cell line cells grow well, are easy to culture, exhibits high tolerance to variations in pH, oxygen levels, and temperature.[10] Primarily, these cells exhibit resistance to human viruses, thereby

[8] Liu, H., Zhou, C., An, J., et al. (2021). Development of recombinant COVID-19 vaccine based on CHO-produced, prefusion spike trimer and alum/CpG adjuvants. Vaccine, 39(48), 7001–7011. https://doi.org/10.1016/j.vaccine.2021.10.066

[9] Zhang, J. H., Shan, L. L., Liang, F., Du, C. Y., & Li, J. J. (2022). Strategies and Considerations for Improving Recombinant Antibody Production and Quality in Chinese Hamster Ovary Cells. Frontiers in bioengineering and biotechnology, 10, 856049. https://doi.org/10.3389/fbioe.2022.856049

[10] Berting, A., Farcet, M. R., & Kreil, T. R. (2010). Virus susceptibility of Chinese hamster ovary (CHO) cells and detection of viral contaminations by adventitious agent testing. Biotechnology and bioengineering, 106(4), 598–607. https://doi.org/10.1002/bit.22723

elevating the safety standards within experimental settings.[11] Their remarkable propensity for high proliferation additionally enhances the efficiency of experiments. They are especially favored for their similarity to human cells in terms of protein glycosylation, assembly and folding processes[12] and post-translational modifications.[13] Given their compatibility with human protein synthesis, CHO cells serve as an effective model for simulating the "healthy" type of cellular uptake of drugs or radiotracers.

Diseased cells have different, pathological protein expression than healthy cells, potentially leading to increased invasiveness and potentially altered drug uptake patterns. This distinction highlights the importance of using affllicted cell models to better understand the efficacy and mechanisms of potential therapeutic strategies, as they provide insights into the specific pathways and targets relevant to the disease condition. PC-3 cells are frequently used in cancer research as the "diseased" cell models to investigate various aspects of tumor biology, including cell proliferation, and response to treatments which differs based on different protein expression.[14] In comparison to other prostate cancer lines, PC-3 cells lack expression of androgen receptors and prostate-specific antigen. PC-3 cell proliferation is not influenced by androgen hormones, making this cell line a prime representation of castration-resistant tumor cells.[15] An important distinction between healthy and diseased cancer cells is the

[11] Wikipedia contributors. (2024, January 7). Chinese hamster ovary cell. In Wikipedia, The Free Encyclopedia. Retrieved 17:27, January 14, 2024, from https://en.wikipedia.org/w/index.php?title=Chinese_hamster_ovary_cell&oldid=1194109159

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[15] Tai, S., Sun, Y., Squires, J. M., Zhang, H., Oh, W. K., Liang, C. Z., & Huang, J. (2011). PC3 is a cell line characteristic of prostatic small cell carcinoma. The Prostate, 71(15), 1668–1679. https://doi.org/10.1002/pros.21383

significantly elevated rate of glucose catabolism in cancer cells. Referred to "the Warburg effect", this heightened metabolic state attributes to cancer cell ability to generate energy (ATP, NADPH) and metabolic compounds (anabolic building blocks) in hypoxic conditions by increasing glycolysis and elevating glucose uptake to support the heightened glycolytic activity needed for daughter cell growth and proliferation. The increased glucose consumption within cancer cells is also advantageous for diagnostic imaging techniques employing glucose-based radiotracers, such as [^{18}F]FDG.[16]

[^{18}F]FDG is a positron-emitting radiotracer containing radioactive 2-deoxy-2-[^{18}F]fluoro-D-glucose. Fluorine-18 ion is created in cyclotron, then converted into [^{18}F]FDG using automated chemistry module.[17] It is produced via either electrophilic or nucleophilic fluorination reaction. As a glucose analog it mimics glucose metabolism in living cells to help diagnosing physiological or pathological processes in which glucose uptake is increased. Physiologically glucose uptake is elevated in the brain, heart, less intensely in the muscles, spinal cord, brown fat etc. [^{18}F]FDG accumulates in pathologically changed tissues with increased glucose demand, including oncological processes such as breast cancer, malignant melanoma and inflammatory conditions, like orthopedic infections and vasculitis.[18] After entering target cell through glucose transporters, [^{18}F]FDG is phosphorylated to [^{18}F]-FDG-6-phosphate and then

[16] Fonseca, J., Moradi, F., Maddalena, L.A., Ferreira-Tollstadius, B., Selim, S., & Stuart, J.A. (2019). Resveratrol integrates metabolic and growth effects in PC3 prostate cancer cells-involvement of prolyl hydroxylase and hypoxia inducible factor-1. Oncology Letters, 17, 697-705. https://doi.org/10.3892/ol.2018.9526

[17] Deng F, Knipe H, DeSai C, et al. F-18 fluorodeoxyglucose. Reference article, Radiopaedia.org (Accessed on 19 May 2024) https://doi.org/10.53347/rID-73056

[18] Ashraf, M. A., & Goyal, A. (2023, August 28). Fludeoxyglucose (18F). In StatPearls. Treasure Island (FL): StatPearls Publishing. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK557653/

is trapped within the cell.[19,20] In comparison to glucose molecule, [^{18}F]FDG contains an added radioactive 18-Fluorine molecule instead of a hydroxyl group at the 2-C position, preventing it from further metabolisation. [^{18}F]FDG is one of the most widely used radiotracers in nuclear medicine (e.g. PET/CT) with high sensitivity (up to 90%[21]) in visual imaging, depicting even minor metabolic deviations before anatomic changes take place. Glucose consumption detection relies on the radioactive decay of 18-fluorine, which has a half-life of 110 minutes.[22] However, it exhibits low specificity (about 70-90%[20]) to differentiate malignant, non-malignant, reactive, infective or inflammatory processes.[23]

[19] Martinkovic, L., Hecimovic, H., Sulc, V., Marecek, R., & Marusic, P. (2014). Chapter Ten - Modern Techniques of Epileptic Focus Localization. In P. Jiruska, M. de Curtis, & J. G. R. Jefferys (Eds.), International Review of Neurobiology (Vol. 114, pp. 245-278). Academic Press. ISSN 0074-7742. ISBN 9780124186934. https://doi.org/10.1016/B978-0-12-418693-4.00010-8.

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[21] Mettler, F. A., & Guiberteau, M. J. (2012). 18F-FDG PET/CT neoplasm imaging. In F. A. Mettler & M. J. Guiberteau (Eds.), Essentials of Nuclear Medicine Imaging (Sixth Edition, pp. 361-396). W.B. Saunders. ISBN 9781455701049. https://doi.org/10.1016/B978-1-4557-0104-9.00011-1.

[22] Ashraf, M. A., & Goyal, A. (2023). Fludeoxyglucose (18F). In StatPearls. StatPearls Publishing, Treasure Island (FL). Retrieved from https://europepmc.org/article/med/32491585#impact

[23] Mbakaza, O., & Vangu, M. (2022). 18F-FDG PET/CT imaging: Normal variants, pitfalls, and artifacts musculoskeletal, infection, and inflammation. Frontiers in Nuclear Medicine, 2. https://doi.org/10.3389/fnume.2022.847810

Aims.

The goal of this thesis encompasses a multifaceted investigation aimed at understanding how minor, but critical aspects affect cellular uptake of radiotracer [^{18}F]FDG.

This thesis seeks to understand the differences in outcomes arising from the utilization of two different cellular uptake measurement techniques to discern the impact of methodological variance on the interpretation and reliability of experimental results.

Determining radiopharmaceutical [^{18}F]FDG cell uptake differences when using media with or without glucose additives. Aiming to elucidate the impact of glucose availability on radiotracer or drug uptake in cells.

Assessing the influence of varying incubation periods (30 min, 60 min, 90 min) on cellular uptake of the radiotracer [^{18}F]FDG thereby providing insights into the optimal temporal parameters for radiotracer uptake studies without compromising cell viability.

By comparing results obtained by using two different membrane plates-hydrophilic polyvinylidene fluoride (PVDF) membrane and glass fiber filter membrane plates, this thesis will provide insights to whether and how different material membranes affect cellular uptake of [^{18}F]FDG.

These diverse and subtle intricacies not only enhances methodologies but also highlights the importance of standardized protocols to maintain consistency across experiments, thus improve the reliability and reproducibility of drug uptake studies.

Materials and Methods.

1. Cell culturing and maintenance.

CHO and PC-3 cell lines were cultured under standardized conditions in a humidified incubator at 37°C with an atmosphere composed of 95% air and 5% CO_2. CHO and PC-3 cells were cultivated as monolayers in Thermo Scientific™ BioLite™ T225 flasks.

CHO cells were grown in Gibco™ F12 Nutrient Mixture (Ham) (1X) media, supplemented with 10% FBS, 1% Penicillin-Streptomycin solution. PC-3 cells were grown in Gibco™ RPMI-1640 media, supplemented with 10% FBS, 1% glutamine, 1% Penicillin-Streptomycin solution. For passage and experimentation, cells were rinsed two times with 10 ml PBS and detached using 4 ml Accutase® (Merck, Sigma-Aldrich) solution. Enzyme was inactivated using double amount (8 ml) of each cell lines` media. Cells were passaged every 3 days.

2. Radiotracer [^{18}F]FDG preparation.

[^{18}F]FDG was produced on site at the Clinical Division for Nuclear Medicine at the Vienna General Hospital via the nucleophilic fluorination reaction. Using cylotron produced Fluorine-18, which was then transferred to an automatic synthesizer (FASTlab, GE HealthCare) for the production of [^{18}F]FDG. The synthesis process took 26 minutes. After hydrolysis and sterile filtration, a [^{18}F]FDG sample was collected for quality control. This included assessing pH and osmolality, as well as radionuclidic purity, radiochemical and chemical purity, sterility testing and subsequent bacterial

endotoxin test. For the cell uptake experiments, [^{18}F]FDG was used within 3-4 h after preparation.

3. [^{18}F]FDG cell uptake using Multiscreen® hydrophilic PVDF membrane 96 well plates and MultiScreen® glass fiber filter membrane 96 well plates.

Both hydrophilic and glass-fiber plates were pre-wet with 100 µl PBS. 90 µl of CHO and PC-3 cells in concentration of 100,000 cells/well were seeded. Cell concentration was calculated by using live cell count obtained with the LUNA-STEM™ automated fluorescence cell counter (Logos Biosystems, Annandale, VA, USA). Then 60 µl media supplemented with 1 MBq/ml of [^{18}F]FDG was added and incubation for one hour at 37ºC followed . After incubation, media was collected, wells were filtrated and washed twice with 100 µl PBS. Plates were then let to air-dry for 5 minutes. By using MultiScreen® Separations System (Millipore, Billerica, MA, USA) consisting of vacuum pump and vacuum manifold, plates were vacuumed to reach higher level of membrane dryeness. After two washings, plate membranes were punched using MultiScreen® punch filter plates. Plate membrane activity was measured using WIZARD2® automatic gamma counter (PerkinElmer Inc., Waltham, MA, USA).

4. [^{18}F]FDG cell uptake using different measurement techniques.

Radiotracer cell uptake can be influenced by the choice of technique used to measure the concentration and activity of uptaken radiotracer. The activity can be assessed through various methods, such as using 96-well hydrophilic membrane plates,

along with conventional uptake measurement technique by using liquid, non-tamed cell.

In the initial phase of the membrane-based measurement technique, cells were incubated for one hour in media containing 0.06 MBq/well [^{18}F]FDG activity, utilizing hydrophilic PVDF membrane plates. After removal of excess liquid through vacuuming, membranes housing the incubated cells, were punched into 5 ml polysterene tubes (nr. 55.1579, Sarstedt, Nümbrecht, Germany) and measured with gamma counter for further analysis.

In comparison, the conventional cellular uptake measurement technique employs free, untamed cells (Figure 1.). In this step, CHO and PC-3 cells were first seeded in 1 ml volume and concentration of 100`000 cells/well in 6-well crystal-grade virgin polystyrene tissue culture test plates (nr. 92406, TPP, Trasadingen, Switzerland). Cell concentration was assessed by quantifying live cells by utilizing LUNA-STEM™ automated fluorescence cell counting system (Logos Biosystems, Annandale, VA, USA). After proliferation of 48h, cell media was changed to 1 ml media containg 1MBq [^{18}F]FDG activity and incubation for one hour in 37^{o}C followed. After incubation period, media was collected and cells were washed twice with 1 ml ice-cold PBS to gather the supernatant. This represents the liquid cell medium, containing unbound [^{18}F]FDG that was not taken up by cells during the incubation period.

To collect cell fraction, 1 ml of T25-trypsin was added, incubated for 5 minutes and inactivated by using twice the volume of media. Both the supernatant and cells from the wells were collected into separate 5 ml Eppendorf tubes and subsequently subjected to gamma counting for quantitative analysis.

Figure 1. Visual representation of membrane-based and conventional cellular uptake measurement technique workflow.

5. [^{18}F]FDG cell uptake differences when using media with or without glucose additives.

Cellular uptake of the glucose-based radiotracer [^{18}F]FDG can differ in media with excess glucose and no-added glucose both for CHO and PC-3 cells. Medias with added glucose were: DMEM/Nutrient mixture F-12 for CHO cells that combines high concentration of glucose, amino acids, vitamins, and glutamine, phenol red, sodium pyruvate[24], and 10% FBS and sodium bicarbonate budder system as an additives; RPMI Medium 1640 (1X) containing glutathione, increased level of vitamins (biotin, vitamin B12, PABA, inositol, choline) and added 1% penicillin-streptomycin, 10% FBS and 1% glutathione was used for PC-3 cells.[25] ThermoFisher DMEM with no glucose was used as a basal media for CHO and PC-3 cells as a comparison. Media was modified

[24] Thermo Fisher Scientific. (n.d.). Product Catalog 11320033. Retrieved from https://www.thermofisher.com/order/catalog/product/11320033
[25] Thermo Fisher Scientific. (n.d.). Product Catalog 11875093. Retrieved from https://www.thermofisher.com/order/catalog/product/11875093

with L-glutamine, phenol red; did not include glucose, sodium pyruvate, HEPES. This cell culture media contains 4x higher amount of amino acids and vitamins compared to other commonly used basal medias. DMEM no-glucose media was prepared with sodium bicarbonate to maintain physiological pH and 10% FBS as a cell growth supplement.[26]

Comparison of cellular uptake was done, incubating 90 µl CHO and PC-3 cells in concentration of 100`000 cells/well in 96-well PVDF membrane plates for an hour in 60 µl of described medias with 1MBq [^{18}F]FDG activity. Cell concentration was calculated by quantifying live cells by utilizing LUNA-STEM™ cell counting system. Plates were then subjected to vacuuming and washing two times using 100 µl PBS, before the membranes were perforated using a punch system. Subsequently, the activity of the plate membranes was quantified using the WIZARD2 gamma counter.

6. Varying incubation period (30 min, 60 min, 90 min) influence on [^{18}F]FDG uptake.

To understand incubation period effect, 90 µl of CHO and PC-3 cells in concentration of 100`000 cells/well were incubated for 30 min, 60 min, 90 min in 96-well PVDF hydrophilic membrane plates. The cell concentration was assessed by quantifying live cell count using LUNA-STEM™ automated fluorescence cell counting system. The quantification of total drug uptake was conducted using a gamma counter.

[26] Thermo Fisher Scientific. (n.d.). Product Catalog 11966025. Retrieved from https://www.thermofisher.com/order/catalog/product/11966025

7. Statistical analysis.

Data analysis was performed using SPSS Statistics 29.0.0 software (SPSS inc., Chicago, IL, USA). The normality of the results was assessed Kolmogorov-Smirnov normality test. For normally distributed data, analyses included two-way ANOVA, unpaired t-tests, and linear regression model when examining the effect of incubation period over time. For non-parametric data, the Mann-Whitney test was used. Statistical significance is indicated as * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$). Data visualization was coducted using GraphPad Prism 8.4.3 (GraphPad Software Inc., California, USA).

Results and Discussion.

1. [^{18}F]FDG cellular uptake in two distinct 96-well plates.

Tests of normality showed that cellular uptake values for both CHO and PC-3 cells did not follow a normal distribution. Therefore, a non-parametric test: Mann-Whitney test was emplyed to compare [^{18}F]FDG uptake in CHO and PC-3 cells cultured on hydrophilic membrane and glass fiber membrane plates.

The analysis of CHO cells indicated a non-significant difference in cellular cellular [^{18}F]FDG uptake between hydrophilic and glass fiber membrane plates (Asymp.Sig. (2-tailed); p=0.93). The mean cellular uptake on hydrophilic membrane plates (n=13) was 0.47 ± 0.25%), with range from 0.14% to 0.95%. In contrast, the mean CHO cellular uptake on glass fiber membrane plates (n=10), was 0.88 ± 1.14% , ranging from 0.25% to 3.69%. This suggests that the type of membrane does not significantly affect the [^{18}F]FDG uptake in CHO cells under the conditions tested.

The results of PC-3 cellular [^{18}F]FDG uptake revealed a statistically significant difference (Asymp.Sig. (2-tailed); p=0.01) when using hydrophilic and glass fiber membrane plates. PC-3 cell mean uptake on hydrophilic membrane plates (n=12) was 1.15 ± 0.29%, varying from 0.61% to 1.54%. However, on glass fiber membrane plates (n=10), the mean cellular uptake was 0.95 ± 1.1%, with min. value 0.32% and max. value 4.00%. This indicates that PC-3 cells show a significantly different uptake of [^{18}F]FDG depending on the membrane type, which could have implications for the cellular interaction with the different substrates.

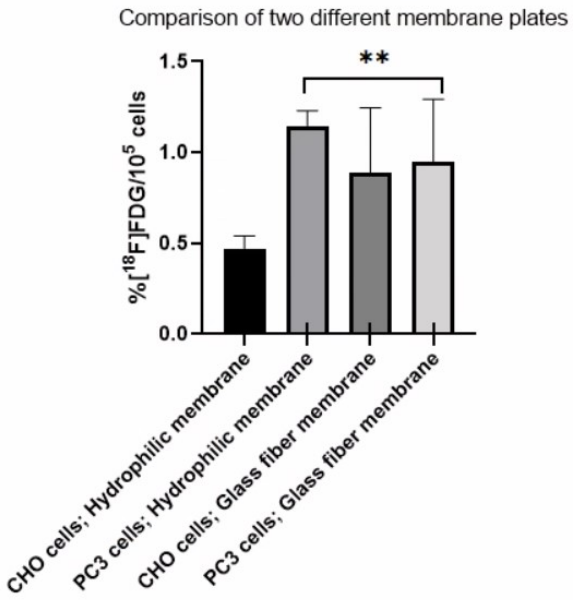

Table 1. Plate membrane material effect on cellular [^{18}F]FDG uptake in CHO and PC-3 cell lines; IP: 60 min

2. Examination of the impact of varying incubation durations (30 min, 60 min, 90 min) on [^{18}F]FDG uptake by cells.

CHO cells: With a 30-minute incubation period, the uptake of [^{18}F]FDG was 0.33 ± 0.21% (min. 0.12%, max. 0.77%) (n=13). Extending the incubation period to 60 minutes resulted in slightly higher uptake values ranging to 0.47 ± 0.25% (min. 0.14%, max. 0.95%) (n=14). Further increasing the incubation period to 90-minutes led to the highest uptake value, ranging to 0.78 ± 0.4% (min. 0.16%, max. 1.56%) (n=14). Statistical analysis using the Repeated Measures ANOVA and a linear regression model showed a significant difference in uptake values across the three incubation periods ($p<0.01$).

Similarly, with a 30-minute incubation period, the uptake of [^{18}F]FDG in PC-3 cells ranged from 0.70 ± 0.23% (min. 0.29%, max. 1.11%) (n=13) to 1.15 ± 0.27% (min. 0.61%, max. 1.54%) (n=13) at 60-minute incubation period. With a 1.5-hour

incubation period, the uptake values further increased to 1.91 ± 0.42% (min. 1.31%, max. 2.58%) (n=14). Repeated Measures ANOVA and a linear regression model confirmed a statistically significant difference in uptake values across the three different incubation periods ($p<0.01$).

Both cell types exhibit increased uptake of [^{18}F]FDG with longer incubation periods. The data indicate a time-dependent increase in [^{18}F]FDG uptake, highlighting the importance of incubation duration in experimental protocols for assessing cellular metabolism. Longer exposure to [^{18}F]FDG allows more efficient uptake and accumulation within the cells. The observed differences were statistically significant, emphasizing the robustness of the time-dependent uptake effect.

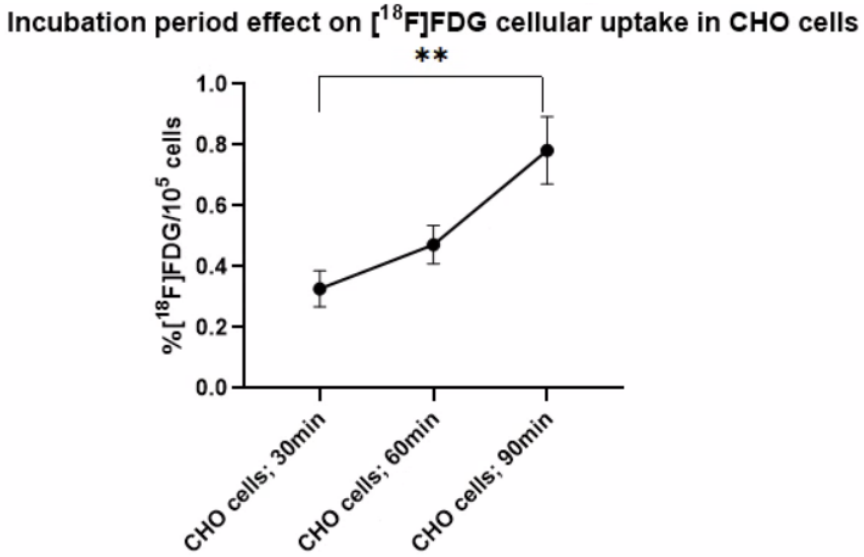

Table 2. Incubation period effect on cellular [^{18}F]FDG uptake in CHO cells.

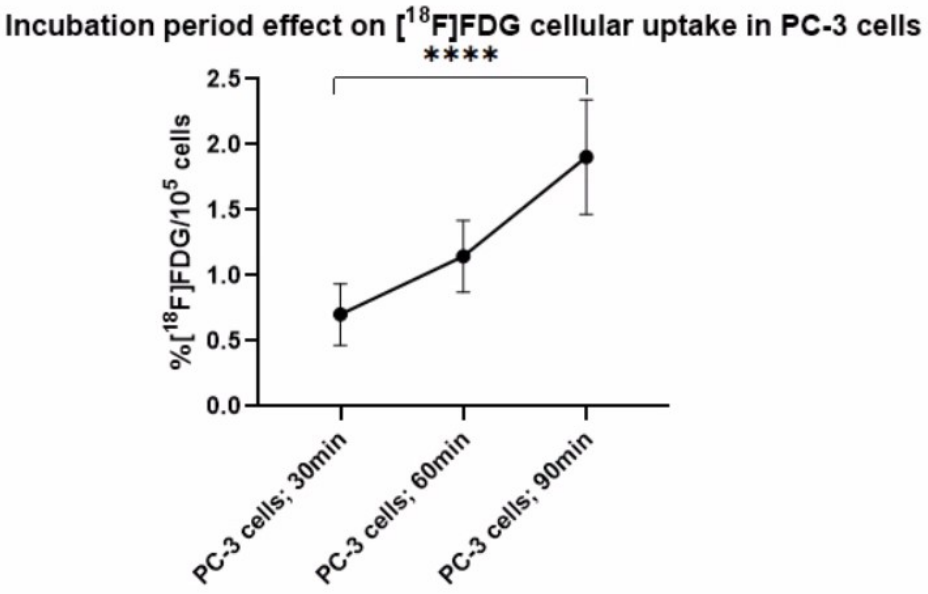

Table 3. Incubation period effect on cellular [18F]FDG uptake in PC-3 cells

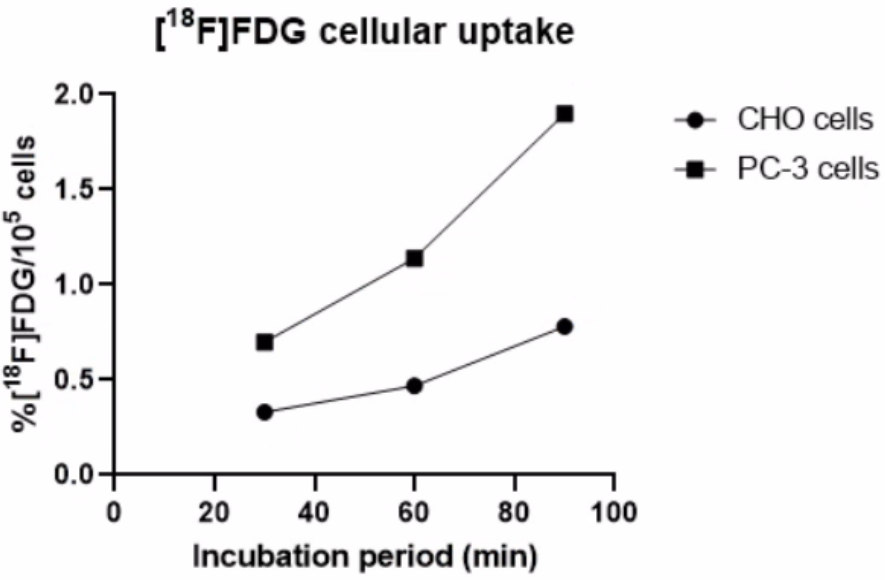

Table 4. Incubation period effect on cellular [18F]FDG uptake in CHO and PC-3 cells.

3. Evaluation of [^{18}F]FDG cell uptake variances when utilizing media with or without glucose additive.

When incubated in DMEM/Nutrient mixture F-12 for CHO cells on hydrophilic membrane plate, which contains a higher level of glucose, the mean cellular [^{18}F]FDG uptake in CHO cells was 0.47 ± 0.25% (min. 0.14%, max. 0.95%) (n=13). However, when using DMEM with no glucose, the mean cellular uptake increased significantly to 4.18 ± 4.21% (min. 0.94%, max. 3.27%) (n=10). Based on Kolmogorov-Smirnov test of normality, CHO cell values in glucose-free media were not normally distributed, therefore non-parametric tests were applied. By using Kruskal-Wallis test, it was confirmed that there is a statistically significant difference ($p<0.001$) in CHO cellular uptake values between the two different media compositions in two different composition medias.

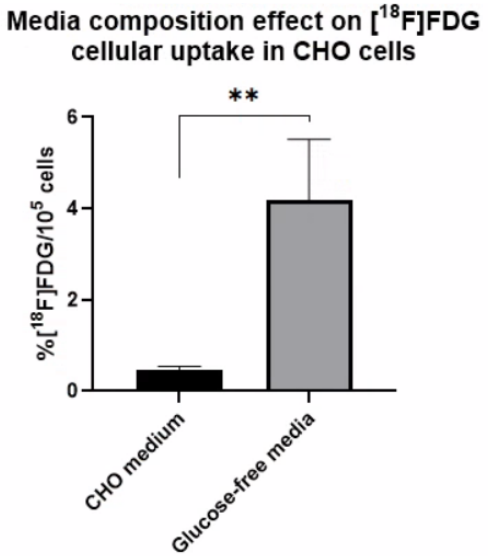

Table 5. Media composition effect on [^{18}F]FDG uptake in CHO cells; Hydrophilic membrane; IP: 60 min

For further exploration, the differences in cellular uptake of [^{18}F]FDG in CHO and PC-3 cells using glucose-containing and glucose-free media were analyzed with the addition of glass fiber membrane plates.

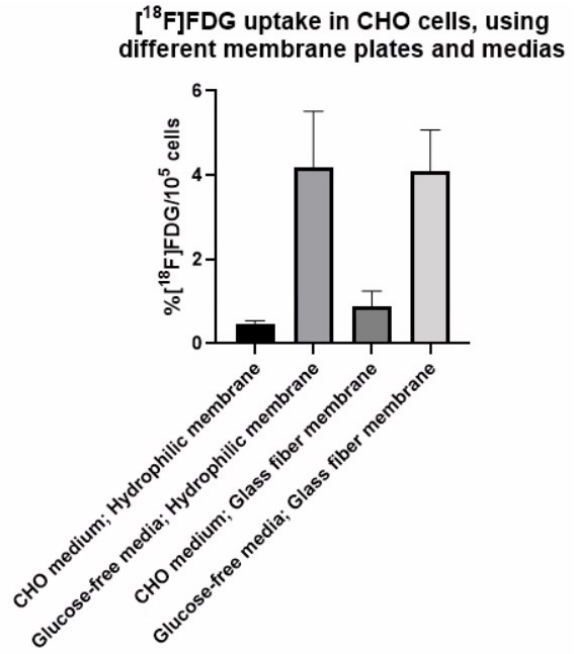

Table 6. CHO cellular uptake in hydrophilic and glass fiber membrane plates using different composition medias; IP: 60 min

When CHO cells were incubated in glucose-containing media and glass fiber membrane plate, the mean cellular uptake reached 0.88 ± 1.14% (min. 0.25%, max. 3.69%) (n=10). Conversely, by implying glucose-free media on glass fiber membrane plates, the mean cellular uptake increased to 4.08 ± 2.97% (min. 0.57%, max. 9.15%) (n=9). By using non-parametric Kruskal-Wallis test, it was shown that there is no statistical difference in [^{18}F]FDG uptake when cultivating CHO cells in glucose-free media on hydrophilic or glass fiber membrane plates (p=0.87).

In conclusion, there is a statistically significant difference in [^{18}F]FDG cellular uptake in CHO cells when using different medias composition, not when using different material membranes.

Similarly, PC-3 mean cellular uptake in RPMI Medium 1640 with added glucose was 1.11 ± 0.28% (min. 0.61%, max. 1.54%) (n=12), while in a media with no additional glucose, the mean cellular uptake reached 6.79 ± 1.4% (min. 5.09%, max.

9.18%) (n=11). By using parametric t-test, it was confirmed that there is a statistically significant ($p<0.01$) difference between cellular uptake when using glucose-containing and glucose-free medias.

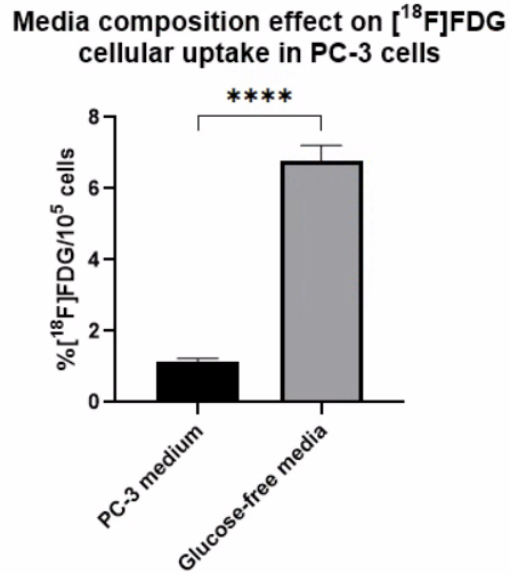

Table 7. Media composition effect on [^{18}F]FDG uptake in PC-3 cells. Hydrophilic membrane; IP: 60min

Effect of media composition on cellular [^{18}F]FDG uptake was analyzed also by using glass fiber membrane plates. Mean cellular [^{18}F]FDG uptake in PC-3 cells incubated on glass fiber plate in glucose-containing media was 0.95 ± 1.1% (min. 0.32%, max. 4%) (n=10). When incubated in glucose-free media on glass fiber membrane plate the mean cellular uptake reached 3.14 ± 2.73% (min. 0.21%, max. 9.2%) (n=11). As the PC-3 cellular uptake values were normally distributed, paired samples test was used. Results showed statistically significant difference ($p=0.04$) when using hydrophilic and glass fiber membrane plates for [^{18}F]FDG cellular uptake.

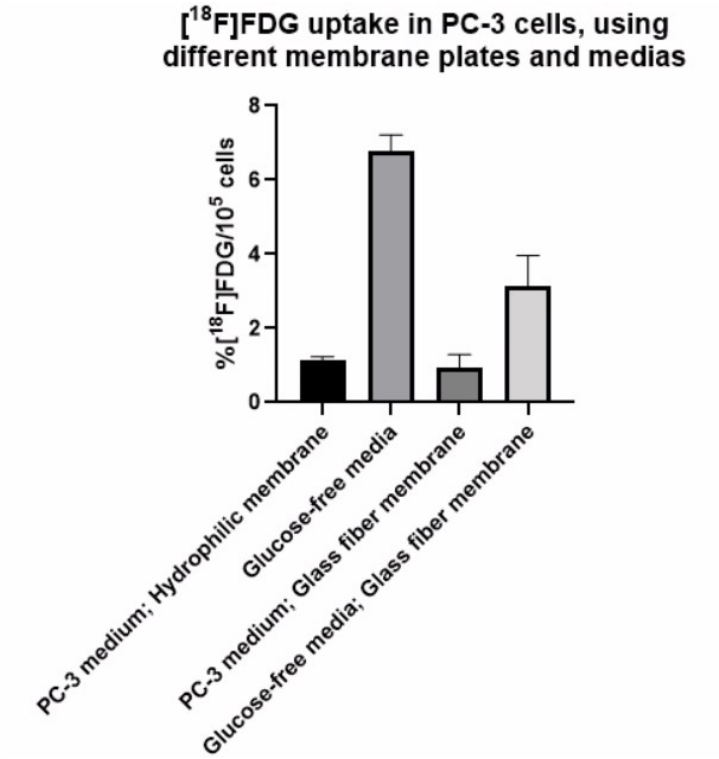

Table 8. PC-3 cellular uptake in hydrophilic and glass fiber membrane plates using different composition medias; IP: 60 min

The difference in drug uptake between CHO and PC-3 cells is more pronounced in glucose-free media. The data strongly suggests that the absence of glucose in the media enhances drug uptake in both CHO and PC-3 cells. Additionally, PC-3 cells tend to uptake more [^{18}F]FDG compared to CHO cells, regardless of media composition, however the absence of glucose significantly enhances radiotracer uptake in PC-3 cells. In glucose-free media, the glucose-bound radiotracer [^{18}F]FDG acts as the sole energy source, leading to higher cellular uptake. Conversely, in media with added glucose, there is a competition between the glucose in media and the radiotracer, resulting in lower level of [^{18}F]FDG uptake.

When combining two independant experimental factors- media composition and plate membrane material- it becomes evident that media composition has a

statistically significant impact on both CHO and PC-3 cellular uptake. In case of membrane material- it had a statistically significant effect on cellular uptake of [^{18}F]FDG in PC-3 cells.

4. [^{18}F]FDG cellular uptake when applying different cellular uptake measurement techniques.

The mean CHO cellular uptake of [^{18}F]FDG when using membrane-based experiment technique was 0.40 ± 0.21% (min. 0.14%, max. 0.73%) (n=14). When using conventional technique- the mean of cellular uptake was 0.14 ± 0.02% (min. 0.11%, max. 0.17%) (n=9). Another experimental factor – different media composition- was applied. The mean CHO cellular uptake when incubating CHO cells in glucose-free media in membrane-based technique was 4.18 ± 4.21% (min. 0.94%, max. 13.27%) (n=10), however when using conventional technique, the mean of cell fraction uptake was 0.97 ± 0.09% (min. 0.89%, max. 0.97%) (n=4). The mean [^{18}F]FDG concentration in CHO supernatant was 10.92 ± 1.87% (min. 7.96%, max. 13.4%). There was a statistically significant difference ($p<0.001$) between the [^{18}F]FDG concentration in supernatant and in the CHO cells. The substantial difference in [^{18}F]FDG levels between the supernatant and the cellular fraction indicates low cellular uptake of the radiotracer during the incubation time. There could be various factors causing low cellular uptake, starting from insufficient incubation conditions, cell health and density, experimental handling procedure factors. However, when compared to results obtained when using membrane-based technique, the cellular uptake of the radiotracer is significantly higher.

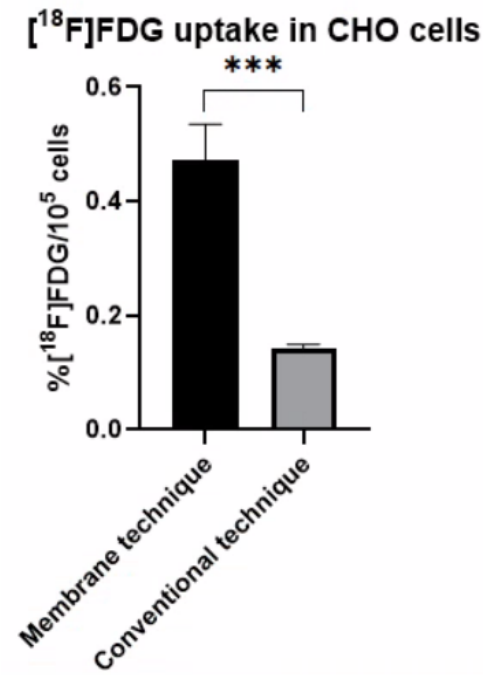

Table 10. [^{18}F]FDG uptake in CHO cells using different measurement techniques; IP: 60 min

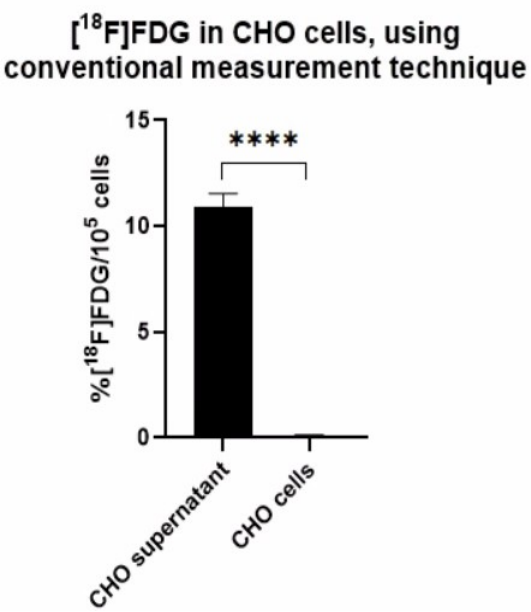

Table 9. Cellular uptake in CHO cells, using conventional measurement technique

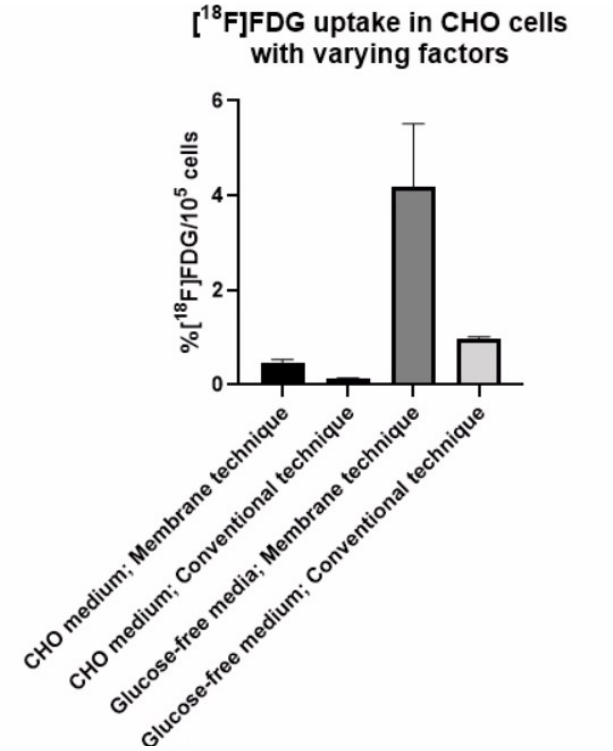

Table 11. Overview of [^{18}F]FDG uptake in presence of two different experiment factors

The mean PC-3 cellular uptake of [^{18}F]FDG when using membrane-based experiment technique was 1.1 ± 0.3% (min. 0.61%, max. 1.54%) (n=9). When using conventional technique- the mean of cellular uptake was 0.28 ± 0.1% (min. 0.2%, max. 0.47%) (n=9). Another experimental factor – different media composition- was applied. The mean PC-3 cellular uptake when incubating PC-3 cells in glucose-free media in membrane-based technique was 3.79 ± 1.4% (min. 1.18%, max. 9.2%) (n=4), however when using conventional technique, the mean of cell fraction uptake was 3.27 ± 0.39% (min. 2.96%, max. 3.81%) (n=4). The mean [^{18}F]FDG level in PC-3 supernatant was 10.2 ± 0.58% (min. 8.5%, max. 12.7%). There was a statistically significant difference ($p<0.001$) among [^{18}F]FDG level in supernatant and in the PC-3 cells. This significant difference of [^{18}F]FDG level in the supernatant and cell fraction suggests that there was low cellular uptake of the radiotracer when using conventional cellular uptake measurement technique.

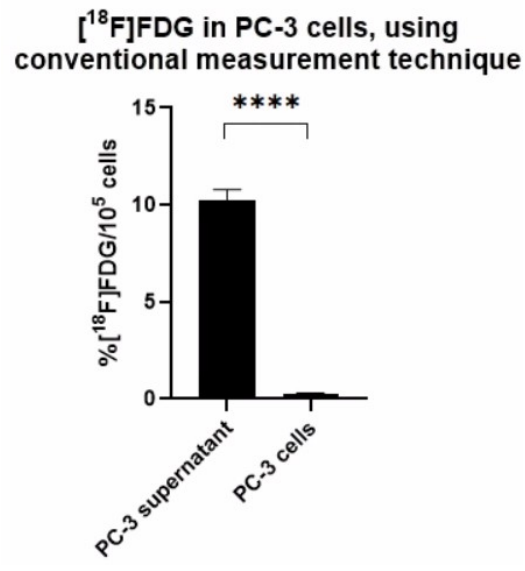

Table 12. Supernatant and cell fraction level [^{18}F]FDG of using conventional measurement technique

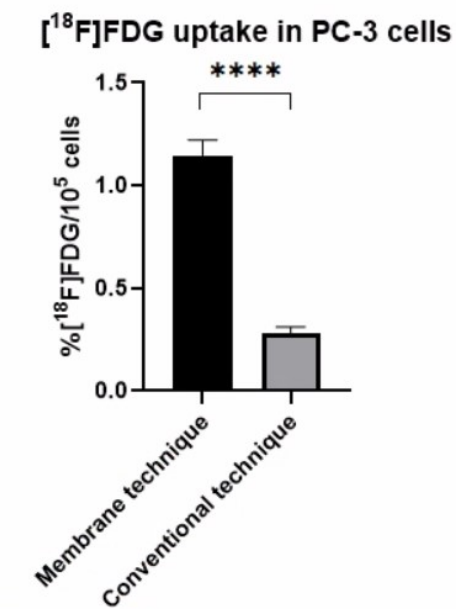

Table 13. [^{18}F]FDG uptake in PC-3 cells using different measurement techniques.

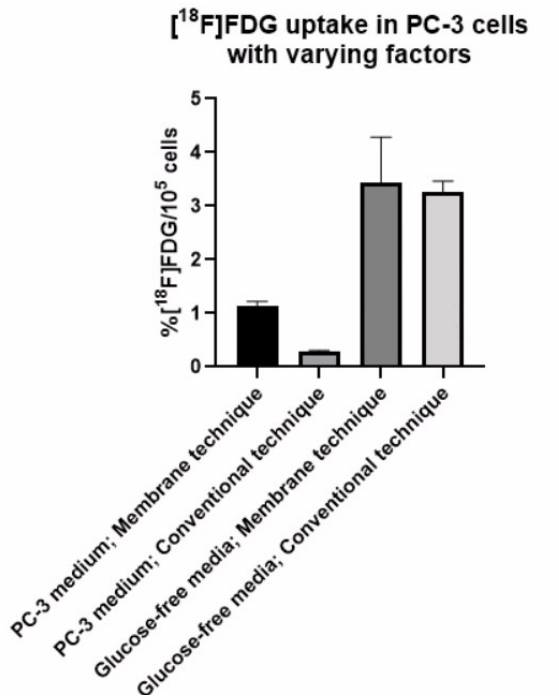

Table 14. Cellular uptake in membrane-based and conventional cellular uptake measurement techniques

Conclusion.

This comparative analysis of plate membrane materials, media compositions, incubation period and experimental methods offers novel perspectives on how subtle differences in experimental setups can influence cellular uptake measurements. Such insights not only contribute to methodological advancements but also highlights the importance of standardized protocols in ensuring consistency and reliability across experiments in various laboratories.

Choice of membrane plate material significantly influences drug uptake, with the hydrophilic PVDF membrane plate demonstrating superior efficacy.

Prolonged incubation periods enhance uptake of [^{18}F]FDG in both CHO and PC-3 cells.

Overall, the absence of glucose in the media enhances drug uptake, with glucose-demanding prostate cancer cells (PC-3) exhibiting higher uptake compared to healthy CHO cells.

When deciding on a technique for measuring radiotracer cellular uptake, the membrane-based technique shows a statistically significant better performance compared to the conventional method.

Declaration of Research Integrity and Good Scientific Practice.

I hereby confirm that this thesis was written independently by myself without the use of any sources beyond those cited, and all passages and ideas taken from other sources are cited accordingly.

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