Single cell radiotracer allocation via immunomagentic sorting (scRadiotracing) to disentangle PET signals at cellular resolution

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ABSTRACT

With great interest, our independent groups of scientists located in Korea and Germany recognized the use of a very similar methodological approach to quantify the uptake of radioactive glucose (18F-FDG) at the cellular level. The focus of our investigations was to disentangle microglial 18F-FDG uptake. To do so, CD11b immunomagnetic cell sorting (MACS) was applied to isolate microglia cells after *in vivo* 18F-FDG injection, to allow simple quantification via gamma counter. Importantly, this technique reveals a snapshot of cellular glucose uptake in living mice at the time of injection since 18F-FDG is trapped by hexokinase phosphorylation without further opportunity to be metabolized. Both studies indicated high 18F-FDG uptake of single CD11b positive microglia cells and a significant increase of microglial 18F-FDG uptake when this cell type is activated in the presence of amyloid pathology. Furthermore, another study investigated noticed that MACS after tracer injection facilitated determination of high 18F-FDG uptake in myeloid cells in a range of tumor models. Here, we aim to discuss the rationale of single cell radiotracer allocation via MACS (scRadiotracing) by providing examples of promising applications of this innovative technology in neuroscience, oncology and radiochemistry.

MAIN TEXT

Potential applications of scRadiotracing in neuroscience

Spatiotemporal alterations of the 18F-FDG-PET signal comprise a well-established read-out in the diagnostic workup of patients with neurological disorders (*1*-*3*). However, the method lacks the cellular resolution to distinguish respective contributions of different cell types to the 18F-FDG uptake. The majority of earlier studies claimed that neuronal activity and neuronal 18F-FDG uptake dominate glucose uptake and consumption in the mammalian brain (*4*). However, several recent studies highlighted a significant contribution role of glial cells for the energy metabolism of the brain (*5*-*7*), which questions 18F-FDG-PET as a pure biomarker of neuronal activation (*8*). We applied immunomagnetic cell sorting after *in vivo* radiotracer injection and brain extraction, followed by subsequent measurement of gamma emission and

cell count in enriched cell fractions to calculate radiotracer uptake per specific single cell (scRadiotracing). Subsequently, our two recent studies exploring scRadiotracing technology identified high microglial 18F-FDG uptake in mouse models with amyloid pathology (*7,9*) (see **Fig. 1** for the workflow). Furthermore, microglial 18F-FDG uptake comprised the most likely reason of elevated 18F-FDG-PET signals in these mice (*7,9*). However, many remaining questions of altered 18F-FDG-PET signals may also be addressed by an analysis of glucose uptake at cellular resolution. Recently, we discovered a reduction of the 18F-FDG-PET signal in a progranulin knock-out mouse model with hyperactivated microglia and in a mouse model with homeostatic microglia (Trem2-knock-out) (*10*). While this finding speaks for reduced brain function in both genotypes, it still remains unclear if progranulin knock-out microglia have lower 18F-FDG uptake or if the net signal is driven by reduced neuronal activity despite higher 18F-FDG uptake of activated microglia (*7,9*). This example illustrates the need to study glucose uptake at cellular resolution. Given the growing evidence for astrocyte-neuron lactate shuttle (*11,12*), it will be of interest to reveal whether increasing neuronal activity (*13*) can stimulate glial 18F-FDG uptake, which could be addressed using scRadiotracing. The presence of such shuttle systems questions the cellular glucose uptake regardless of the individual glucose consumption of different cell types, which is undeniably high in active neurons (*14*). In this regard, there could be an imbalance between the cellular localizations of glucose uptake and energy consumption. Importantly, scRadiotracing after 18F-FDG injection could be specifically used to allocate glucose uptake, while other tracers could be used to track metabolites of aerobic and anaerobic glycolysis at the cellular level. Furthermore, uptake mechanisms during acute stimulation of glucose consumption by physiological (*15*) or pharmacological interventions could be deciphered by scRadiotracing. To this end, task related stimuli could be applied together with resurgent functional PET methodology (*16*) and scRadiotracing in experimental models. scRadiotracing could be applied after brain extraction at the time-point of maximal stimulation, determined by the functional PET read-out, to track the changes of cellular 18F-FDG uptake in contrast to unstimulated conditions.

As another example in the field of neuroimaging, tau-PET tracers emerged as valuable biomarkers for the differentiation of tauopathies from controls (*17*). However, the translation of *in vitro* tau-PET tracer binding to an *in vivo* signal is still under debate and the detailed cellular sources of autoradiography and tau-PET signal elevations remained unclear. Hence, our novel scRadiotracing approach could be used to calculate tau-PET tracer uptake at cellular resolution of single neurons and astrocytes in models of tauopathies in order to close this knowledge gap. As a general note, brain subregion analyses by scRadiotracing are also feasible as long as the product of the cellular yield and the tracer abundance exceed the detection limit of the gamma counter. In this regard, we successfully dissected the hippocampus to study region specific 18F-FDG uptake in mice with amyloid pathology (*9*). In tauopathies, this feature could be used to compare single cell radiotracer uptake of regions with high and low tau abundance.

Potential applications of scRadiotracing in neurooncology and oncology

The novel combination of tracer injection and immunomagnetic cell sorting could also facilitate dedicated analysis of tumor cells in experimental models of brain tumors together with analysis of specific immune cell fractions and tumor surrounding cells such as neurons and ependymal cells. This could be highly valuable since the target of several tracers for glioblastoma imaging, including the 18kDa translocator protein (TSPO), is not restricted to a single cell type. Determining the radiotracer uptake of TSPO ligands at cellular resolution in brain is of general interest since the target is not specifically expressed by microglia cells but also by tumor cells, astrocytes, endothelial cells and neurons (*18*). Since blood-brain-barrier disruption is often questioned as a strong influencer of PET tracer signals in brain tumor imaging, scRadiotracing could also act as a proof of cellular radiotracer allocation. Here, the magnitude of radiotracer uptake per cell could be multiplied with respective absolute cell numbers to investigate if the entire PET signal is explained by cellular sources.

Apart from brain tumors, the aforementioned oncological investigation (*19*) already applied scRadiotracing in a wide range of tumor models, including renal cell carcinoma, colorectal carcinoma (CT26, MC38), and breast cancer. Importantly, the authors did not only employ 18F-FDG but also used 18F labelled glutamine to disentangle it's metabolism in tumor cells and the tumor microenvironment (*19*). This shows that scRadiotracing is not limited to 18F-FDG and highlights the broad range of potential scRadiotracing applications in experimental oncology. For example, prostate specific membrane antigen (PSMA) radiotracers are preferred to target prostate cancer cells because they exhibit very low glucose consumption and therefore cannot be detected by 18F-FDG-PET. However, PSMA is not only expressed in prostate cells, but can be found in several other tissues such as nonprostatic epithelial cells, other neoplastic cells, and tumor-associated neovasculature. PSMA uptake was also associated to inflammatory and infectious processes (*20*). Thus, scRadiotracing could be used to differentiate PSMA uptake in prostatic cancer cells from others and to avoid pitfalls in prostate cancer diagnostics. The same applies to the diagnostics of well differentiated neuroendocrine tumors (NETs) such as gastroenteropancreatic NETs and meningeomas using radiolabeled somatostatin receptor (SSTR) ligands. The importance of NET diagnostics and therapy is underpinned by an interventional multicenter phase III clinical trial (NETTER) to compare peptide receptor radionuclide therapy using 177Lu-DOTA^{0}-Tyr^{3}-Octreotate to treatment with high dose Octreotide LAR in patients with metastasized or locally advanced, inoperable, SSTR positive, midgut carcinoid tumors. However, SSTR expression is not exclusive for NET or meningioma cells. Tracer uptake was also observed for inflammatory pathologies including cardiovascular disease and ischemia, and various other benign and malignant tumors (*21*). scRadiotracing has the potential to resolve such inconclusive results by use of back translation in experimental models. This may help to determine off-target sources and prevents false positive findings. In cases with borderline SSTR expression, dual scRadiotracing may also be suitable to elucidate if SSTR radioligands or 18F-FDG are better suited for follow-up PET imaging of the individual patient.

Potential applications of scRadiotracing in radiochemistry and radiotracer development

scRadiotracing might also be a versatile tool in tracer development to investigate cell specific uptake of acutely isolated cells when compared to cell culture where metabolic activity of cells may be altered. In general, *ex vivo* radiopharmaceutical research methods mostly rely on macroscopic samples for quantification in a gamma counter or on autoradiography blocking experiments including correlation studies with immunohistochemistry staining. However, such techniques do not investigate tracer enrichment on the cellular level. Nevertheless, this is of particular interest, when the specificity of a novel tracer has to be explored or when the radioactivity distribution is to be assigned to specific cell types. In neuroinflammatory tissue, the discrimination of tracer accumulation in different microglia phenotypes could be of eminent importance. Such approaches could support the development of specific ligands for homeostatic and disease-associated microglia which would facilitate monitoring of therapeutic agents that modulate distinct microglia phenotypes. In principle, scRadiotracing could be applied to any radiotracer binding to an intracellular target. However, it is questionable if ligands of membrane bound targets on the surface of cells also qualify for scRadiotracing analysis. Upstream cell processing promises a gentle mechanical and enzymatic dissociation preserving cell integrity and surface epitopes, but it has to be proven if high affinity binders withstand hydrolytic treatment. This also applies to subsequent downstream applications beyond quantification in a gamma counter. In this respect, ligands showing a high internalization rate, may be most likely applicable to scRadiotracing analysis.

Methodological limitations and considerations

First, we note the difficulty to quantify the uptake of a whole cell population via scRadiotracing. The procedure of cell dissection and harvesting may over- or underestimate the proportion of viable cells of the brain or in specific regions (22), which hampers extrapolation to absolute cell numbers. Cell proportions can also be influenced by proliferation and cell loss which lead to subsequent alterations in cell density. Thus, scRadiotracing

facilitates robust calculation of radiotracer uptake per cell, but extrapolation to the whole fraction is erroneous. One possibility to establish a full allocation model of radiotracer uptake per cell type and fraction could be established by simultaneous light sheet microscopy (*23*). Light sheet microscopy offers the possibility to quantify the absolute amount of cells per cell type in a 3-dimensional volume, i.e. by analysis of a subsample of the target.

Unlike well-established nearly irreversibly bound 18F-FDG, all non-18F-FDG radioligands may suffer from higher instability during the scRadiotracing procedure. For instance, the binding stability of a TSPO ligand to the TSPO complex at the mitochondrial membrane could decrease during the scRadiotracing procedure. This also accounts for current tau-ligands like 18F-PI-2620 which is characterized by a decrease of target-bound radiotracer over time in 4-repeat tauopathies (*24*).

Loss of processes and synapses potentially impact quantitative results of scRadiotracing. 18F-FDG in neuronal synapses comprises one important example of missed radiotracer in the scRadiotracing workflow (*7,9*). Optimization of the dissociation procedure has the potential to further enhance the accuracy of scRadiotracing. In similar regard, consideration of live and dead cells may stabilize scRadiotracing results since cells with leaky membranes can be excluded.

Cell separation after TSPO tracer injection was also proposed by a recent study using a similar technique of fluorescence activated cell sorting (FACS) to determine the cellular source of a 125I labelled single photon emission computed tomography (SPECT) TSPO ligand (125I-CLINDE) (*25*). Here, the long half-life of 125I offered the opportunity to conduct the experiments with less time constraints. When comparing both approaches, MACS systems can be installed relatively simple within a radiation protection controlled area and the higher cell yield is another advantage of MACS over FACS, which even allows to perform proteomic analyses (*26*). On the other hand, FACS offers the advantage of direct separation of genetically determined fluorescent cells (i.e. GFP) which cannot be achieved via MACS. Furthermore, specific cell populations, such as homeostatic or disease associated microglia, can be selected

via FACS gating, when discriminative antibodies are used. Considering the stability of radiotracer binding once more, there is a need to investigate the impact of emitted energy during cell sorting via FACS, which could be of high relevance for tracers bound at voltage channels. Head-to-head comparisons of both approaches will be required to allow recommendations on a preferential use of MACS or FACS systems for scRadiotracing.

scRadiotracing in human tissue after *in vivo* or *in vitro* tracer application comprises another promising methodological variant (*25*). *In vitro* application of radiotracer to tissue allows to investigate small amounts of tissue which do not yield a sufficient signal-to-noise ratio when the tracer is applied *in vivo* (i.e. prior to surgery of tumors). However, scRadiotracing after tracer application *in vivo* could be used to validate *in vitro* scRadiotracing in the same tissue when amounts are large enough. Furthermore, blocking with cold ligands could be performed to test for the specificity of cellular radiotracer binding using *in vitro* scRadiotracing. As a limitation, we note that the extensive work flow (see **Fig.1**) likely restricts scRadiotracing to an experimental setting but prevents it from use in clinical routine at the current stage.

In summary, we highlighted some of the broad range of highly demanded applications for the novel scRadiotracing workflow to elucidate tracer uptake mechanisms and their underlying cell biology.

Noteworthy

- Cellular sources of 18F-FDG and other PET imaging radiotracers are poorly understood (page 2, line 11-17)
- Immunomagnetic cell sorting after radiotracer injection (scRadiotracing) facilitates determination of *in vivo* radiotracer uptake per specific single cell (page 2, line 18-22)
- scRadiotracing is of high interest when radiotracer targets are expressed and pathologically altered on different cell types, such as applicable for 18kDa translocator protein (TSPO) (page 4, line 8-12)

Figure Caption

Figure 1 - Workflow of single cell radiotracer allocation via immunomagnetic sorting (scRadiotracing) to determine microglial 18F-FDG uptake in brain at cellular resolution: After tracer injection into the tail vein, the brain is removed upon a tracer specific uptake period. Following the generation of a single cell suspension, immunomagnetic cell separation is used to separate fractions of enriched cells and their depleted counterparts, which contain bound radioactivity. Fluorescent labeling, gamma counting and flow cytometry are used to calculate radioactivity per cell as the primary read-out. Time necessary to complete each experimental step is indicated together with the summed timed during the workflow. p.i. = post injectionem. CD11b is used to detect microglia.

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Disclosure

The authors do not indicate a conflict of interest