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*Hypoxic markers*

# Imaging hypoxia after oxygenation-modification: Comparing [$^{18}$F]FMISO autoradiography with pimonidazole immunohistochemistry in human xenograft tumors

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## Abstract

***Purpose*: Hypoxia is one of the reasons for radiation therapy resistance. Positron emission tomography using $^{18}$F-labeled misonidazole ([$^{18}$F]FMISO) is a non-invasive method of imaging tumor hypoxia. Aim of this study was to validate [$^{18}$F]FMISO against the clinically most widely used hypoxic cell marker pimonidazole under different oxygenation conditions.**

***Materials and methods*: One human head and neck squamous cell carcinoma (SCCNij3) and two human glioblastoma (E102 and E106) xenograft tumor lines were studied after injection of [$^{18}$F]FMISO and pimonidazole. Control mice were compared with a second group breathing carbogen to reduce tumor hypoxia and with a third group with clamped tumors to increase hypoxia. Tumor sections were analyzed on a phosphor imaging system and consecutively stained immunohistochemically (IHC) for visualization of pimonidazole. Pixel-by-pixel analysis was performed and the hypoxic fraction, obtained after segmentation of the pimonidazole signal, was related to the mean optical density of [$^{18}$F]FMISO and pimonidazole.**

***Results*: A moderate pixel-by-pixel correlation between [$^{18}$F]FMISO autoradiography and pimonidazole IHC was found for the control tumors, after carbogen breathing and after clamping for SCCNij3. For E102 and E106, mean signal intensities for pimonidazole significantly decreased after carbogen breathing and increased after clamping, mean [$^{18}$F]FMISO signal intensities increased significantly after clamping and a significant correlation between the hypoxic fractions and the mean [$^{18}$F]FMISO signal intensities was found.**

***Conclusions*: [$^{18}$F]FMISO autoradiography and pimonidazole immunohistochemistry can both be used to visualize treatment induced changes in tumor hypoxia. However, the response to these modifications differs widely between xenograft tumor lines.**

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Hypoxia is present in most solid tumors due to impaired oxygen and nutrient supply to the tumor [12]. Hypoxia is associated with an increased likelihood of locoregional recurrence and distant metastases. Furthermore, hypoxia decreases the response to radiation therapy and to several cytotoxic agents, leading to poor treatment outcome and prognosis [11,20,21,23]. To overcome hypoxia-induced radioresistance, several treatment modifications have been developed, such as the use of hyperoxic gas breathing under normal or hyperbaric conditions, vasoactive drugs and hypoxic cell sensitizers. These modifications often lead to increased toxicity and morbidity for the patient. Furthermore, not all patients benefit from this treatment intensification. For instance, patients with non-hypoxic tumors will not gain by hyperoxic gas breathing while still experiencing the increase in side effects. Therefore, careful selection of patients for these intensified treatment modalities is necessary.

Traditionally, the oxygen partial pressure ($pO_2$) in accessible human tumors, e.g., the uterine cervix and lymph nodes, is measured using the invasive oxygen-sensitive Eppendorf histograph needle electrode [2,18,21–23]. Hypoxia can also be detected by visualization of bioreductive hypoxic cell markers in tumor sections. The two

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exogenous hypoxia markers approved for patient studies are EF5 and pimonidazole [8,24]. Bio-reduction of these 2-nitroimidazoles strongly increases at $pO_2$ levels below 10 mm Hg. Recently, the predictive value of hypoxia detected by pimonidazole for treatment outcome was shown in advanced stage head and neck cancer [16]. The use of endogenous hypoxia-related markers, such as carbonic anhydrase IX (CAIX) or the glucose transporters (Glut-1 and -3), has been extensively investigated for this purpose. However, results thus far are conflicting [4,15,28,30].

Although these hypoxic cell markers are excellent tools for quantification of hypoxia at the microregional level, these assays cannot be used to monitor changes of tumor hypoxia over time. Promising non-invasive imaging modalities permitting repeated measurements include magnetic resonance imaging techniques and positron emission tomography (PET) of radiolabeled bio-reductive markers [17,27]. Currently, the clinically most widely used hypoxia PET-tracer is [$^{18}$F]fluoromisonidazole ([$^{18}$F]FMISO) [7,17,25,29]. However, [$^{18}$F]FMISO cannot be validated as a hypoxia marker at the microregional level as no antibody is available until present.

This study was designed to evaluate the potential usefulness of [$^{18}$F]FMISO for the detection of changes in tumor hypoxia in three human xenograft tumor lines after carbogen (95% $O_2$, 5% $CO_2$) breathing and clamping and compare the results with the well-established hypoxic cell marker pimonidazole.

## Materials and methods

### Animals and tumor models

The human head and neck squamous cell carcinoma xenograft tumor line SCCNij3, and the human glioblastoma tumor lines E102 and E106 were used for these experiments. Viable 1 $mm^3$ tumor pieces were implanted subcutaneously in the abdominal flank of athymic BALB/C nu/nu mice and tumors were used for the experiments at a diameter of 8—9 mm. All mice were kept in accordance with institutional guidelines. All experiments were approved by the Animal Experiments Committee of the Radboud University Nijmegen.

### [$^{18}$F]FMISO synthesis

[$^{18}$F]FMISO was synthesized according to the method described by Lim and Berridge [19]. The 1-(2′-nitro-1′-imidazolyl)-2-*O*-tetrahydropyranyl-3-*O*-toluenesulfonyl-propanediol (NITTP) precursor was obtained from ABX GmbH (Radeberg, Germany) and [$^{18}$F]fluoride was obtained from BV Cyclotron VU (Amsterdam, The Netherlands).

Radiochemical yield (decay corrected) was always higher than 40% and radiochemical purity was always higher than 98%. Specific activity at end of synthesis was higher than 50,000 GBq/mmol.

### Experimental setup

Mice were stratified into three groups of 5—6 animals, based on tumor size; one group served as control, the other groups were studied either under carbogen breathing or clamping. At the start of the experiment the control animals were injected i.v. with 37 MBq of the hypoxia PET-tracer [$^{18}$F]FMISO in 0.2 mL. After 5 min the hypoxia marker pimonidazole hydrochloride (1-[(2-hydroxy-3-piperidinyl) propyl]-2-nitroimidazole hydrochloride; Natural Pharmacia International, Research Triangle Park, NC) was injected i.p. at a dose of 80 mg/kg. One hour after injection of [$^{18}$F]FMISO the animals were killed by cervical dislocation. The mice in the carbogen group started breathing carbogen (95% $O_2$, 5% $CO_2$; Hoek Loos, Schiedam, The Netherlands) 5 min before injection of [$^{18}$F]FMISO and continued until the animals were killed 1 h later. In the clamping group, 30 min after administration of [$^{18}$F]FMISO, tumors were clamped for 30 or 60 min until killing of the animals 1 or 1.5 h after administration of the PET-tracer, respectively. Immediately after killing the mice, tumors were removed and snap frozen in liquid nitrogen. Within 30 min, frozen tumor sections of 5 μm thickness were cut from the central part of the tumor and sections were used to detect the [$^{18}$F]FMISO signal on the phosphor imaging system (PPI).

### Autoradiography

From each tumor a central 5-μm section was cut and mounted on poly-L-lysine coated slides. Tumor sections were exposed to a phosphor imaging screen overnight. The screen was scanned in a phosphor imager system (Molecular Imager GS363, Bio-Rad Laboratories) at a pixel size of 100 × 100 μm. Images were processed with Quantity One software (version 4.5.2, Bio-Rad Laboratories, Hercules CA). The same tumor section was then used for immunohistochemical staining and analysis of pimonidazole labeling.

### Immunohistochemical staining for hypoxia

Between all consecutive steps of the staining procedure, the sections were rinsed three times for 5 min in 0.1 M phosphate-buffered saline (PBS, Klinipath, Duiven, The Netherlands), pH 7.4. The sections were first fixed in cold acetone (4 °C) for 10 min. After re-hydration of the tissue sections in PBS during 20 min, they were incubated with rabbit anti-pimonidazole (gift J.A. Raleigh) diluted 1:1000 in primary antibody diluent (PAD, Abcam, Cambridge, UK) for 30 min. Sections were then incubated with donkey anti-rabbit-Alexa488 (Molecular Probes, Leiden, The Netherlands) diluted 1:400 in PBS for 30 min. Finally, sections were mounted on Fluorostab (Organon, Boxtel, The Netherlands).

### Immunohistochemical image acquisition

All tumor sections were analyzed using a digital image analysis system as described previously [26]. After scanning whole tissue sections, gray scale images for pimonidazole were obtained and subsequently converted into binary images.

Thresholds for segmentation of the fluorescent signals were interactively set above the background level. Binary images were used to calculate the hypoxic fraction (HF); tumor area positive for pimonidazole relative to the total tumor area. Areas of necrosis were excluded from the analyses.

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