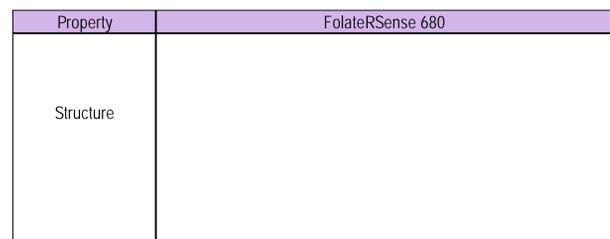


Abstract

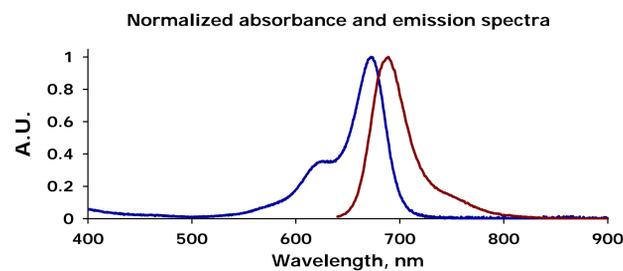
Folic acid (vitamin B9) is an essential nutrient required by eukaryotic cells for survival that is acquired and internalized via folate receptors (FR) on the cell surface. In particular, it is the highly metabolic cells that upregulate FR, and many different human tumor cells, including ovarian, breast, cervical, renal, colorectal and nasopharyngeal cancer cells show significant upregulation as compared to normal tissues. As such, folic acid has been successfully exploited as a cancer specific targeting moiety for the efficient delivery of chemotherapeutic agents, drug carriers, photosensitizers and diagnostic reporters. Critical to the success of such agents is the determination of the level of FR expression for a given tumor, since weak FR-expressing cancers will not respond well to folate-targeted therapies. There is therefore a need for specific and quantitative imaging agents and methods for the determination of FR expression in vivo.

Optical imaging in the near-infrared (NIR) range allows efficient penetration of photons through living tissue and minimizes interference from tissue autofluorescence. Combined with quantitative fluorescence molecular tomography (FMT), NIR fluorescent agents have emerged as invaluable tools for quantitative, deep tissue imaging across a range of important areas of disease research including oncology. We have developed a new, near-infrared fluorescent folate targeted agent, FolateRSense™ 680 (FR680), and used it to noninvasively quantify FR expression in vivo by FMT of mouse tumor xenografts. Three different cell types, KB, HeLa and HT-29, with varying degrees of FR expression were employed in the present studies. In vitro, the binding of FolateRSense 680 to each cell type was visualized by fluorescence microscopy and compared with FITC-labeled anti-FR antibody and the binding was quantified by flow cytometry which indicated a K_d of approximately 5 nM. Specificity of the agent was demonstrated by blocking of the signal by addition of an excess of unlabeled folic acid. In vivo quantification was performed by injection of 2 nmol of FR680 into nude mice bearing KB, HeLa or HT-29 tumor xenografts and imaging by FMT at 4 to 24 h post injection. KB tumors showed a high level of FR expression in the two tumors (300-400 total pmol, as quantified by FMT), with little fluorescence in other tissues except the kidneys at 4 h post injection in a biodistribution study. Consistent with the in vitro profile, HeLa and HT-29 tumors had less tumor fluorescence. All tumor cell lines showed significant (70-80%) knockdown of signal by co-injection of the mice with unlabeled folic acid and ex vivo colocalization with FR antibody on tumor slices, confirming the in vivo specificity of the agent. Thus, we have demonstrated the ability of FolateRSense 680 to quantify FR expression both in vitro and in vivo and, combined with FMT, to noninvasively distinguish between high and lower FR expressing tumors.

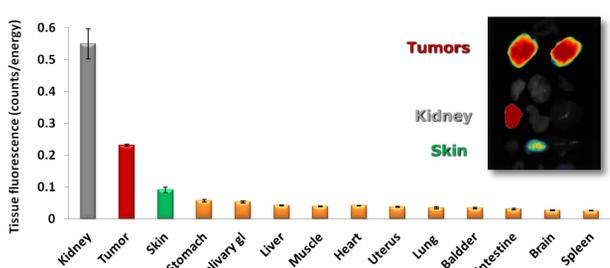
1 Description of the Agent

Property	FolateRSense 680
Structure	
MW	1606 g/mol
Absorbance (max)	670 nm
Emission (max)	686 nm
ϵ (670 nm)	210,000 M ⁻¹ cm ⁻¹

FR680 was synthesized by coupling the NIR fluorochrome VivoTag-680 (VT680) selectively to the γ -carboxylate of folic acid followed by preparative HPLC purification and characterization by LCMS.

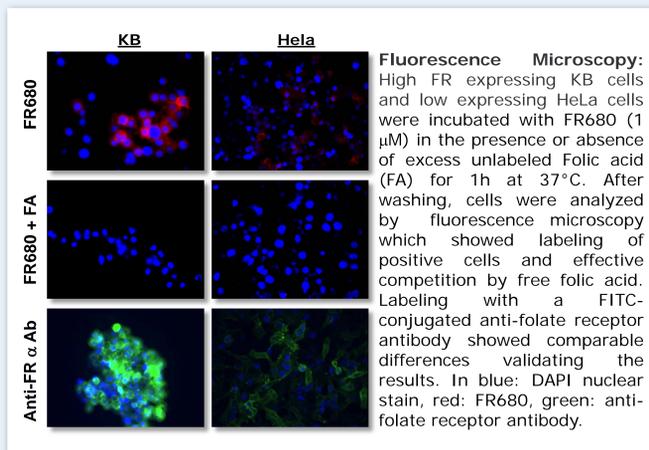


2 Biodistribution

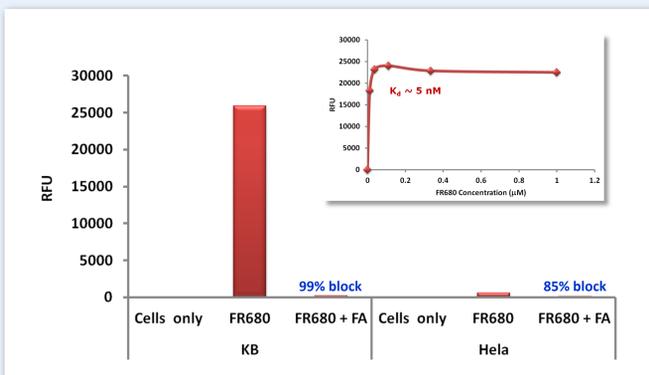


KB cells were injected into the mammary fat pads of Nu/Nu female mice. Once tumors were established, mice were injected intravenously with 2 nmoles FR680. Tissues were collected 4h later and fluorescence assessed by planar imaging (ex vivo). Mean counts/energy for each tissue were determined as a measure of tissue brightness. High kidney signal indicates a predominant renal clearance of FR680.

3 Fluorescence Microscopy

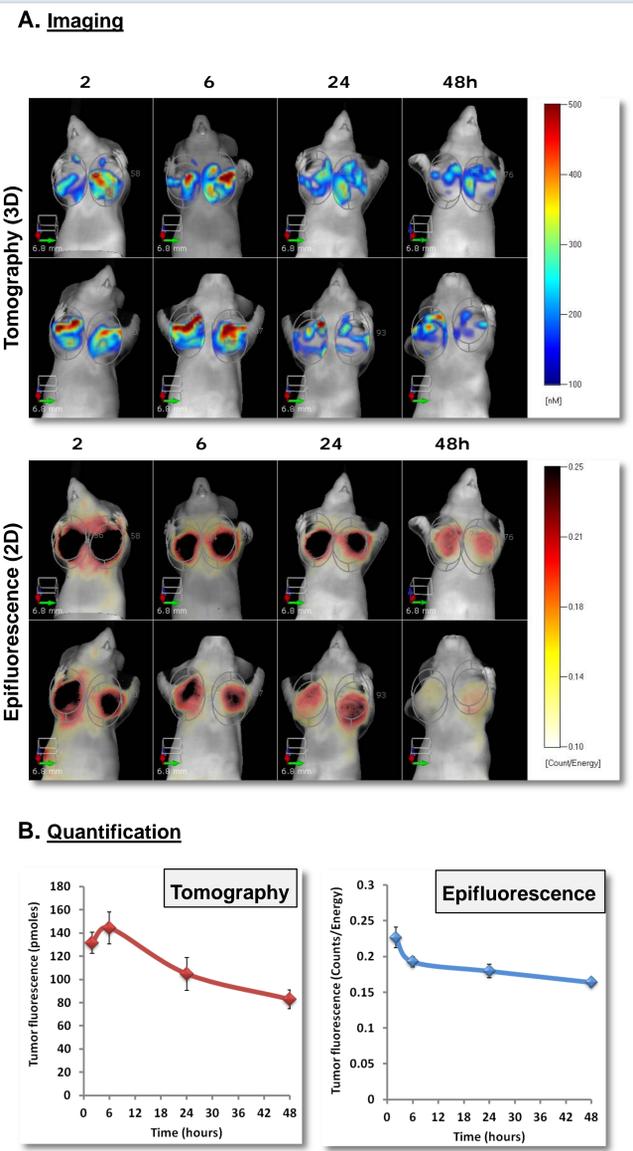


4 Flow Cytometry



Flow cytometry: Flow cytometry confirmed significantly higher (40X) uptake of FR680 by high FR expressing KB cells over low expressing HeLa cells and blocking of signal by 99% and 85%, respectively, with FA. Binding experiments with KB cells over a range of concentrations indicated a dissociation constant (K_d) or approximately 5 nM (inset).

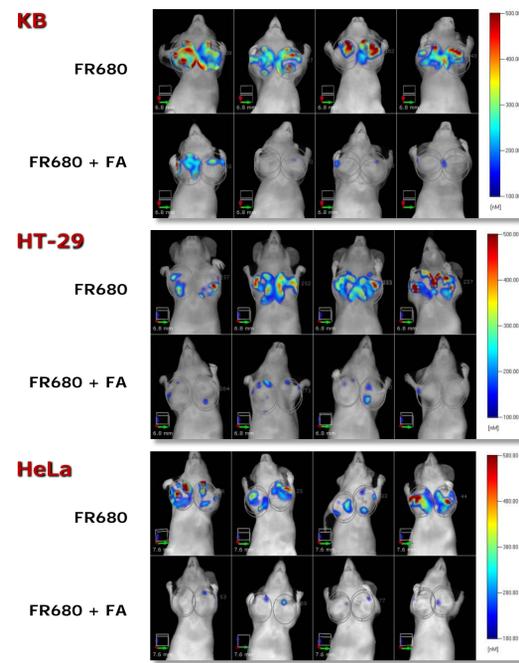
5 Imaging Kinetics in KB Tumors



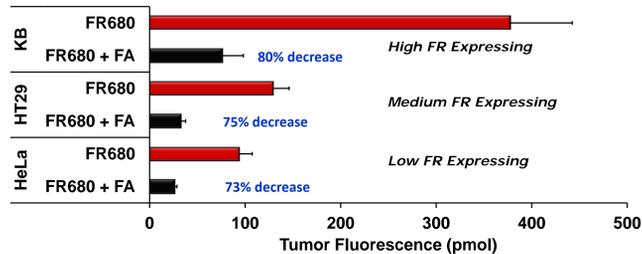
A. Nu/Nu female mice, bearing KB tumors implanted on the upper mammary fat pads, were injected IV with 2 nmoles FR680 and imaged on the FMT™ 2500LX both tomographically (top) and by epifluorescence (bottom) at different time points. Two representative mice are shown across multiple time points. **B.** Quantification of the tumor site signal (pmoles by tomography) and relative tumor brightness (mean counts/energy by reflectance) were determined.

6 FMT Imaging and Quantification

A. Imaging

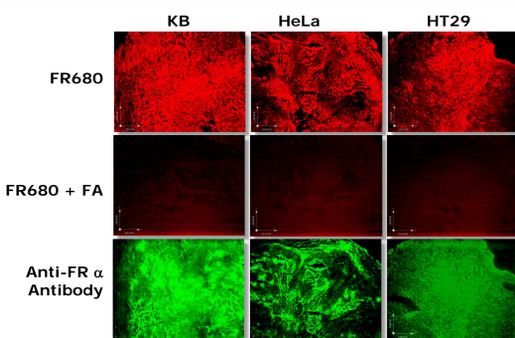


B. Quantification



KB, HeLa or HT-29 tumor bearing mice were injected with FR680 in the absence or presence of 100x excess folic acid and imaged tomographically 4h later. **A.** Shown are 4 representative mice per group. **B.** Quantification of FR680 signal in tumors. Results show folate-specific blockade of FR680 accumulation in the tumors, even in the case of low-expressing tumor lines. This is attributed to the presence of FR-expressing inflammatory cells.

7 Tissue Localization



KB, HeLa or HT-29 tumor-bearing mice were injected with FR680, with or without excess FA. Tumors were excised 4h later and snap frozen. Fluorescence microscopy shows labeling of tumor cells (red), effective competition by free FA and comparison to FITC-conjugated anti-FR antibody (green). 25x final magnification. Acquisition time was the same for FR680 with and without FA. Results show excellent agreement between in vivo FR680 label and ex vivo tissue staining.

Summary

Folate receptors are a family of cell surface receptors for the essential vitamin folic acid (folate, vitamin B9), and these receptors are upregulated in highly metabolic cells due to the high need of this vital nutrient. High receptor expression can be measured in a variety of cancers (including breast, ovarian, lung, kidney, colon, and brain) as well as in tumor associated macrophages. Folic acid offers a high affinity targeting ligand, and researchers have used a variety of different labels in order to image folate receptor upregulation in vivo. Optical imaging of a near infrared fluorescent-labeled folic acid (FolateRSense 680) offers a safe and easy means for non-invasive preclinical imaging, in real time and in vivo. The results presented here demonstrate the imaging and validation of this agent in vitro, in vivo, and ex vivo in three different tumor xenograft models expressing different levels of folate receptor.

References

- Leamon CP, Low PS: Folate-mediated targeting: from diagnostics to drug and gene delivery. Drug Discov Today 2001, 6: 44–51
- Vlahov IR, Leamon CP: Engineering Folate—Drug Conjugates to Target Cancer: From Chemistry to Clinic. Bioconjugate Chem. 2012, 23:1357-1369..