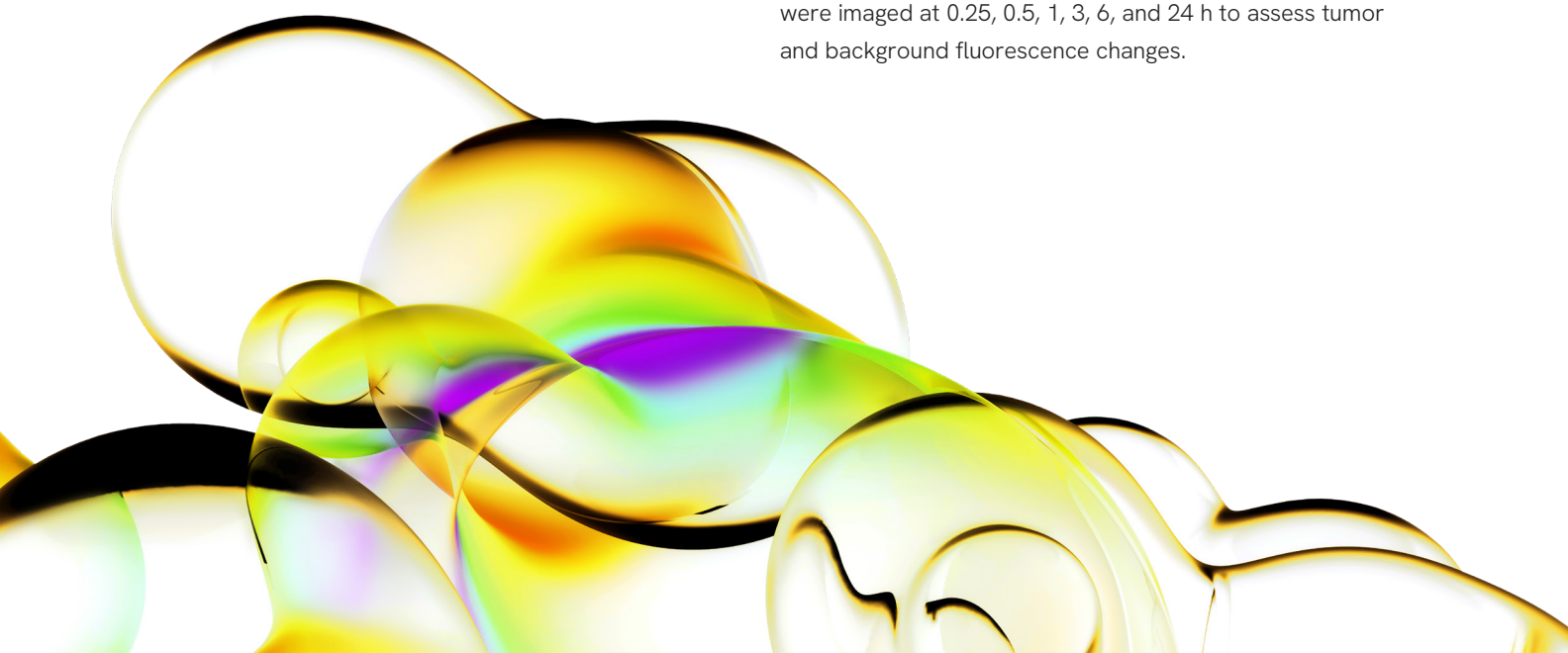
Characterization of near infrared fluorophores for their potential in non-specific tissue accumulation.

The design of targeted probes used in fluorescence imaging *in vivo* requires precise control over the drug-like properties of prepared conjugates. The specificity of fluorescent probes is intended to be imparted solely by the targeting moiety selected (peptide, protein, small molecule drug, or antibody). However, any other modification to the overall construct can have effects on both the general biodistribution and the specificity of targeting the relevant biomarker. Even the fluorophore reporter used can have an impact on specificity and biodistribution, with some fluorophores known to be somewhat prone to non-specific retention. This is especially true in sites of tumor growth or inflammation.

In this application brief, we examine the 'stickiness' of Revvity's fluorophores in comparison to those of a leading competitor. Furthermore, we demonstrate the impact an overly sticky fluorophore can have on image quality and information deduced from such images.

Comparison of IVISense™ fluorophore stickiness with a commonly used alternative fluorophore

To test and select fluorophores for their adverse accumulation properties, the free acid form of the fluorophore are injected mice bearing tumors. By using the free acid version of the fluorophores, we can avoid unwanted labeling of serum proteins *in vivo*. This is the approach that was used here. We looked at four different fluorophores, IVISense 680, IVISense 750, a competitor's 680, and a competitor's 750. Following intravenous injection with fluorophores, tumor-bearing mice were imaged at 0.25, 0.5, 1, 3, 6, and 24 h to assess tumor and background fluorescence changes.



In vivo optical imaging using the IVIS™ system demonstrated that Revvity's IVISense 680 and 750 fluorophores exhibit superior performance in mouse cancer models compared to commonly used competitor fluorophores. Collectively, Figures 1, 2, and 3 clearly demonstrate that the IVISense fluorophores have minimal accumulation in tumors and result in low background signal across tissues. This is further supported by quantitative analysis, which reveals that competitor fluorophores show slower clearance from mouse tissue and a tendency to accumulate non-specifically within tumors. In contrast, the IVISense fluorophores are cleared quickly and do not preferentially localize to tumor tissue, suggesting a higher specificity and lower potential for off-target effects.

Figure 1: Images obtained clearly show that Revvity's IVISense 680 and 750 fluorophores have little accumulation in tumors and low general background tissue signal. In contrast, the commonly used competitor fluorophores tend to accumulate signal nonspecifically in tumor regions.

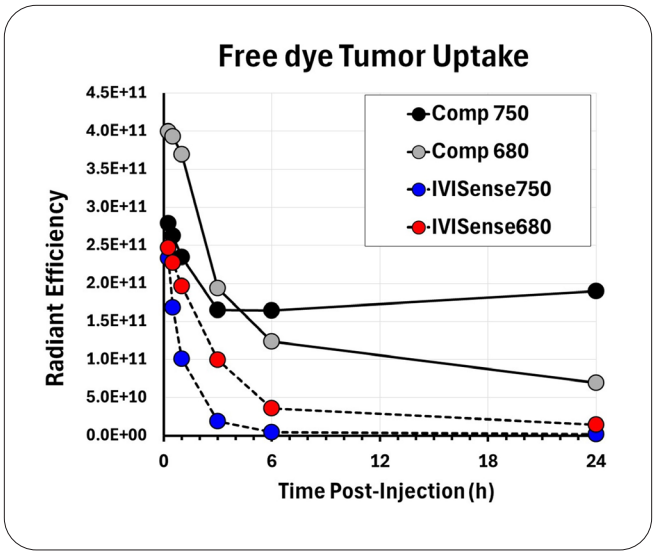


Figure 2: **Quantitative assessment of tumor fluorescence** shows slower clearance kinetics for competitor fluorophores from mouse tissue with clear accumulation within tumors. Revvity IVISense fluorophores, in contrast show quick clearance and no preferential localization to tumor tissue.

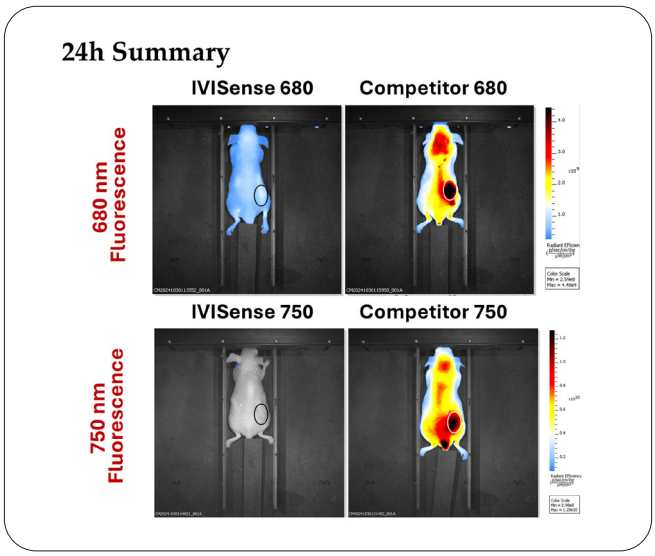


Figure 3: **Evaluation after 24 hours.** Images for 680 nm and 750 nm fluorophore signal in tumor-bearing mice are shown with matching scales for 680 nm and 750 nm fluorophores, respectively.

Can a "sticky" fluorophore alter the biodistribution of a non-targeted, neutral protein?

A typical protein of 30-150 kDa will be conjugated with NIR fluorophores at a ratio of 1-3 fluorophores per protein molecule. This can yield a brightly labeled protein with minimal interference of binding sites or protein function. To assess the impact of fluorophore property on protein biodistribution, we selected an untargeted protein that exhibits minimal selective tissue biodistribution when injected *in vivo*. Neutravidin (MW 60 kDa) is a deglycosylated version of chicken avidin, that has reduced lectin binding and a near-neutral pH (pH 6.3), minimizing non-specific interactions with the negatively charged cell surfaces. Neutravidin still has lysine residues that remain available for derivatization or conjugation.

We labeled Neutravidin at different conjugation ratios with Competitor 680 fluorophore (2, 4, and 5 fluorophores per protein molecule) and compared it to IVISense 680 conjugated at 4 fluorophores per protein molecule. Twenty-four hours post-injection, it was obvious that as low as two Competitor 680 fluorophore molecules were required to cause unwanted tumor accumulation of a Neutravidin conjugate (Fig. 4).

This study suggests that as little as 2 fluorophores with unwanted tumor accumulation properties are sufficient to increase overall tissue background signal as well as yield disproportionate accumulation within a tumor xenograft.

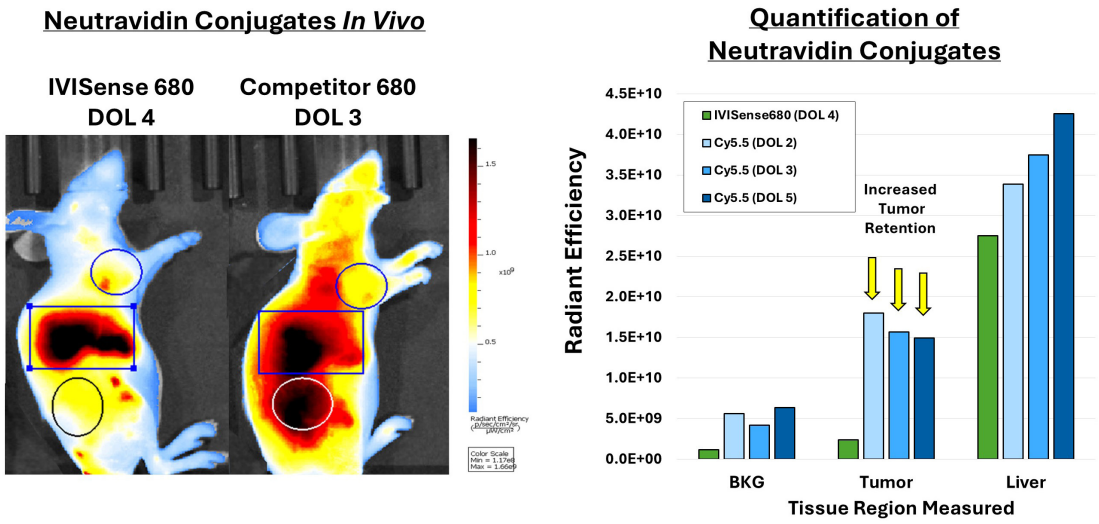


Figure 4: ***In vivo* Imaging (a) and quantification (b) of Neutravidin conjugates.** As little as 2 fluorophores with unwanted tumor accumulation properties are sufficient to increase overall tissue background signal as well as yield disproportionate accumulation within a tumor xenograft.

Summary

The "stickiness" of a fluorophore is a critical factor in achieving high-quality, reliable, and informative *in vivo* images of mouse cancer models. It directly impacts the signal clarity, accuracy of tumor detection, duration of imaging, and ultimately, the biological insights gained from the studies. We have shown here that the "stickiness" of a fluorophore can affect the biodistribution, even in a non-targeted neutral protein. Furthermore, we have demonstrated that Revvity's IVISense fluorophores are less sticky than their leading competitor, leading to less background noise and higher image quality.