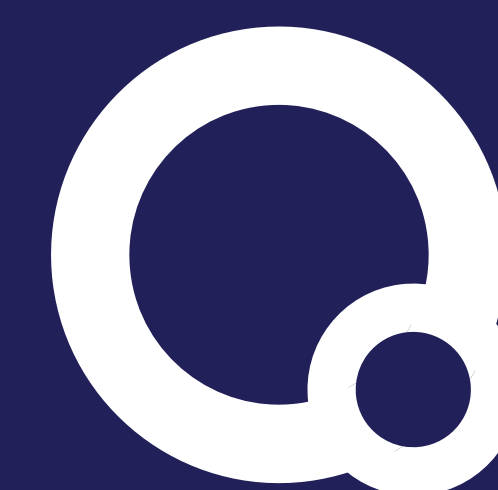


The Use of Discrete Polyethylene Glycol (dPEG®) Linkers to Reduce Non-Specific Staining in Immunofluorescence

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SUMMARY

Objective

To develop dye-dPEG[®]x-antibody conjugates that are brighter and show less non-specific staining than standard conjugates without the dPEG[®]x linker

Initial Targets

Fluorescein-dPEG[®]₁₂-Goat Anti-Rabbit IgG (FL-dPEG[®]₁₂-GAR)

- ◆ At least 20 FL-dPEG[®]₁₂ can be added to a Rabbit IgG with little or no self quenching. In the case of FITC addition, self-quenching is severe after ~6 dyes are added
- ◆ In a tissue IHC assay, FL-dPEG[®]₁₂-GAR specifically binds to its antigen and shows dramatically less background staining than FITC-GAR.

Fluorescein-dPEG[®]₁₂-Goat Anti-Mouse IgG (FL-dPEG[®]₁₂-GAM)

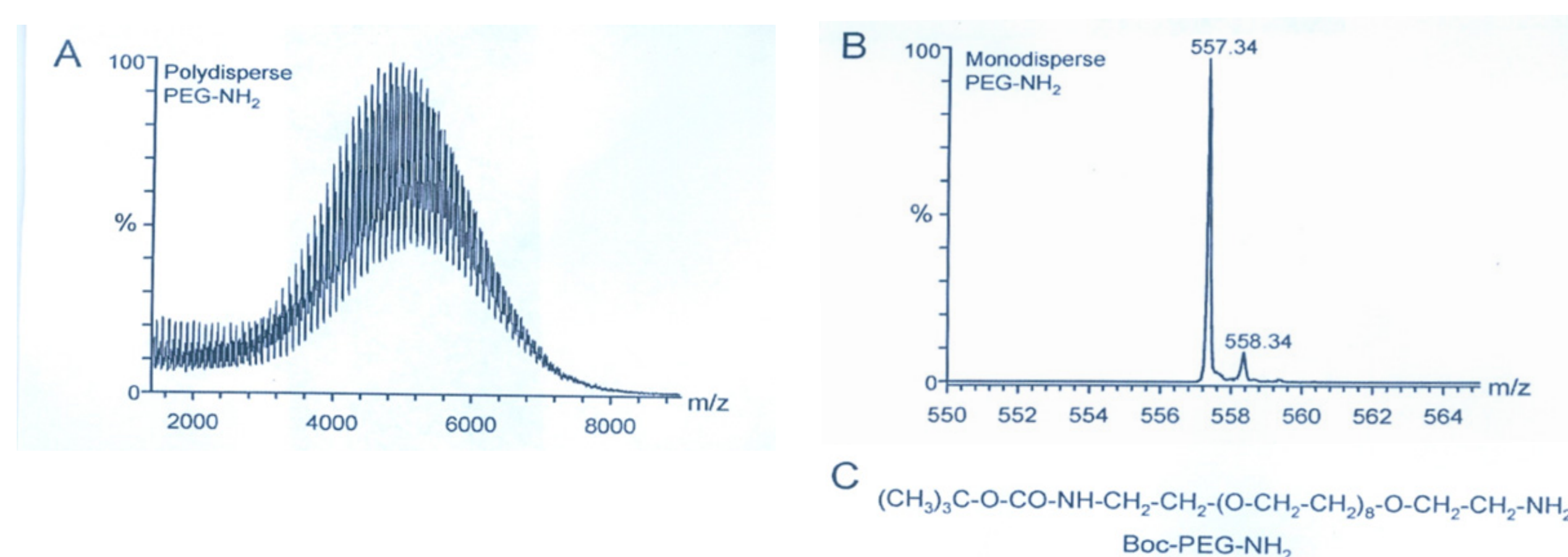
- ◆ In a cell based assay, FL-dPEG[®]₁₂-GAM showed specific binding and a much lower background than FITC-GAM

R-Phycoerythrin-dPEG[®]₂₄-Streptavidin (RPE-dPEG[®]₂₄-SA)

- ◆ Multiple RPE-dPEG[®]₂₄-SA Conjugates have been made with different size distributions
- ◆ These conjugates are currently being tested in Luminex and Flow Cytometry applications

Figure 1.

A dPEG[®] is a single molecule PEG compound!
Comparing conventional polymeric PEGs* to a dPEG[®]



* ...the inherent nature of the linear construct actually forces them to be much TOO big...and is probably the source of "competitive biology"!

Figure 2.

Heterobifunctional dPEG[®] Linker Chemistries

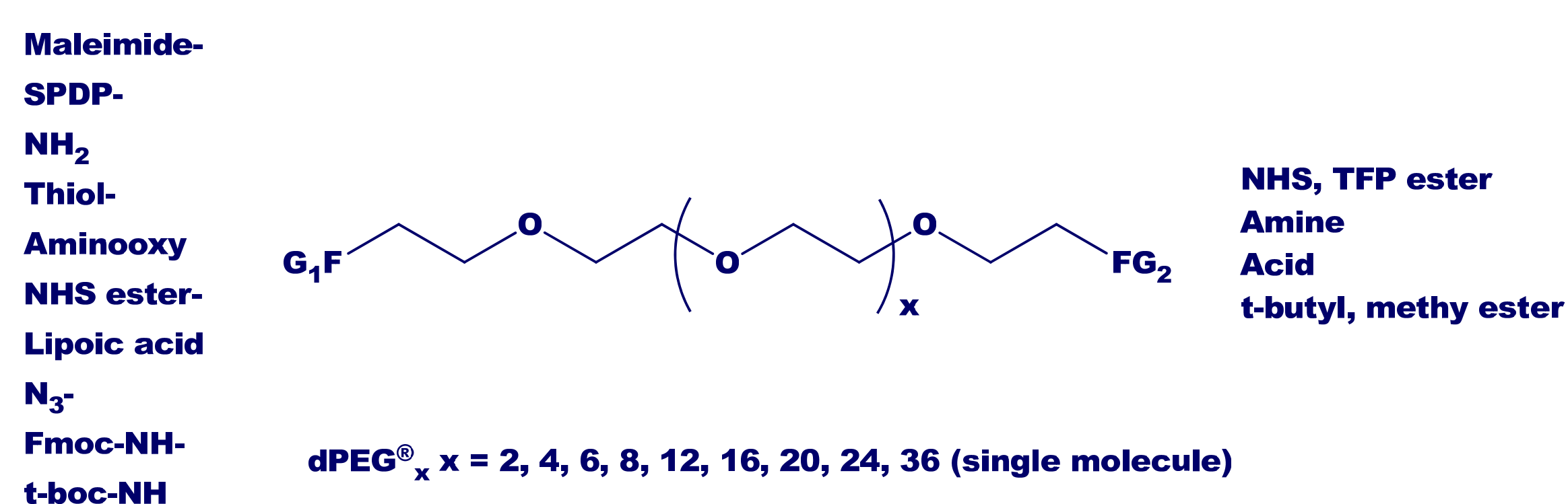


Figure 3.

Preparation of Fluorescein-dPEG[®]₁₂ Labeled Antibodies

Synthesis of Fluorescein-dPEG[®]₁₂-NHS

- Method for Preparing a dye-dPEG[®]₁₂-NHS from any maleimide activated dye
 - Reduce SPDP-dPEG[®]₁₂-NHS with an equimolar amount of DTT at pH 5
 - React with an excess of Dye-Maleimide
 - Reagent is used to label antibodies without purification
- Fluorescein-dPEG[®]₁₂-NHS will be available shortly as a Quanta BioDesign catalog item

Antibody Labeling

- Fluorescein-dPEG[®]₁₂-NHS is reacted with antibodies at pH 7.2 for 1 hour
- Unreacted labeling reagent is removed via SEC
- Very high degrees of fluorescein substitution can be obtained with little or no self quenching

Figure 4.

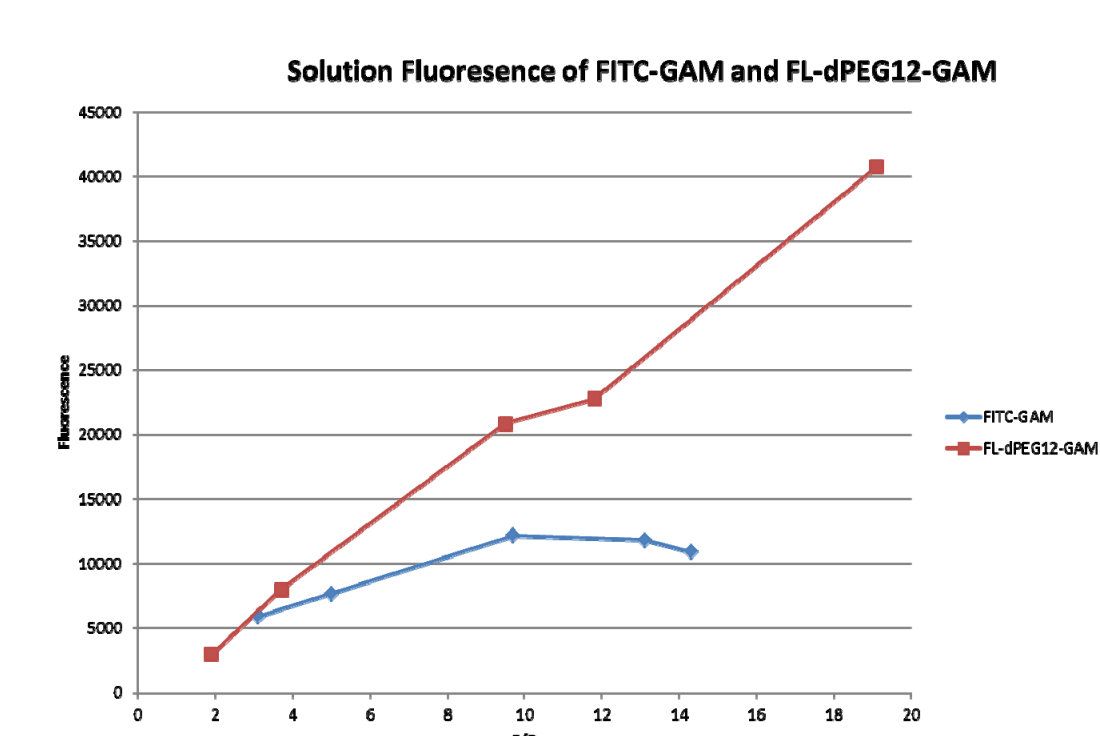


Figure 5. IHC Performed on Paraffin Embedded Tissue by an Industrial Collaborator

- ◆ Rabbit 1^o MAb against non-disclosed cancer antigen
- ◆ HRP-GAR 2^o Ab provides effective staining of cancer cells
 - Collaborator is developing a multi-color fluorescent assay to measure several markers simultaneously
 - FITC-GAR 2^o Ab gives unusable backgrounds
 - FL-dPEG[®]₁₂-GAR 2^o Ab has low background and clear CA staining

Figure 6. HRP-GAR 2^o Ab

Arrow Indicates CA Positive Tissue

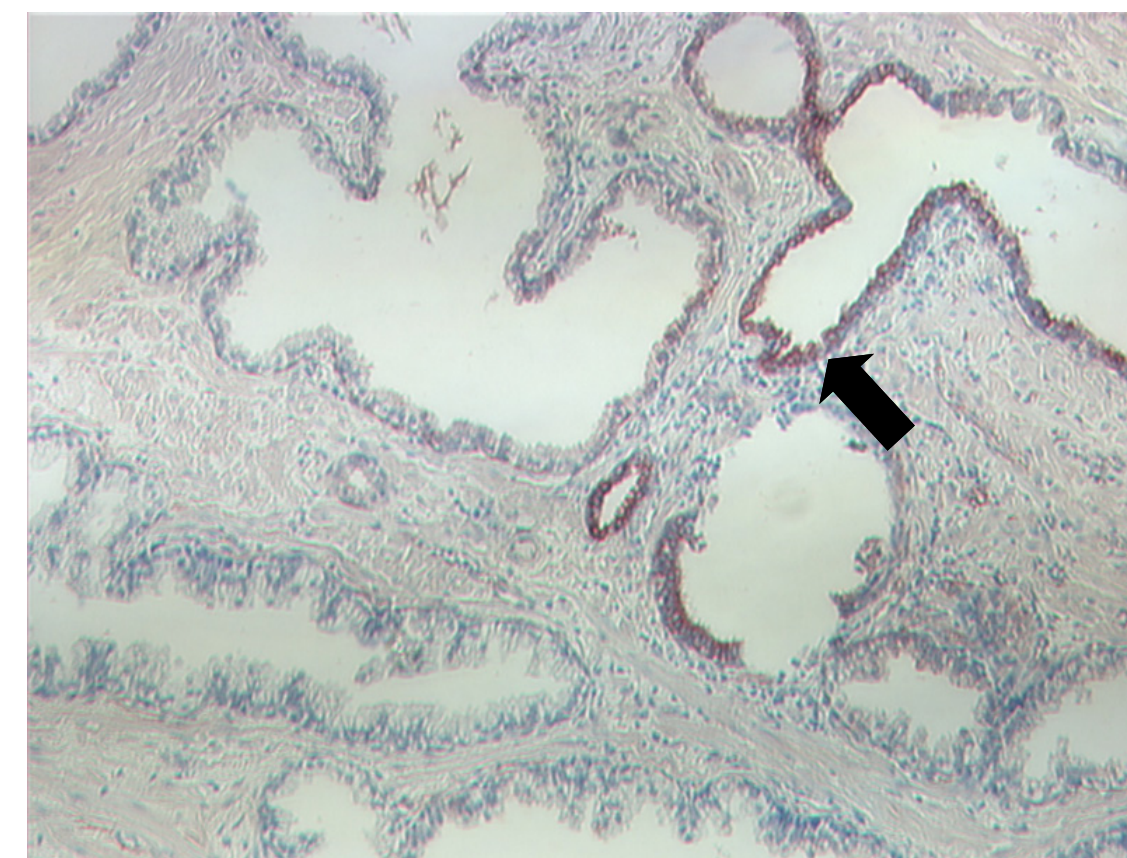
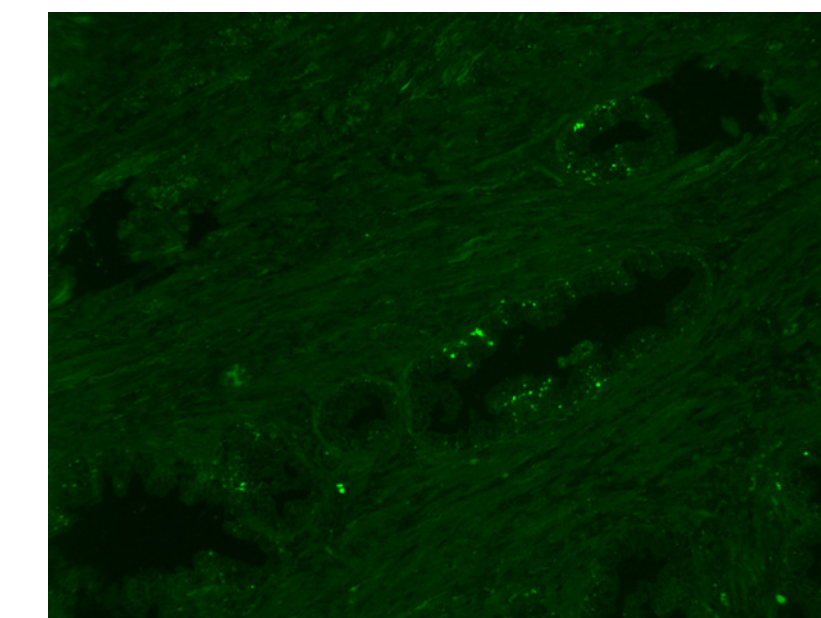


Figure 7.

Rabbit Primary Antibody

Secondary Ab = FITC-GAR



Secondary Ab = FL-dPEG[®]₁₂-GAR

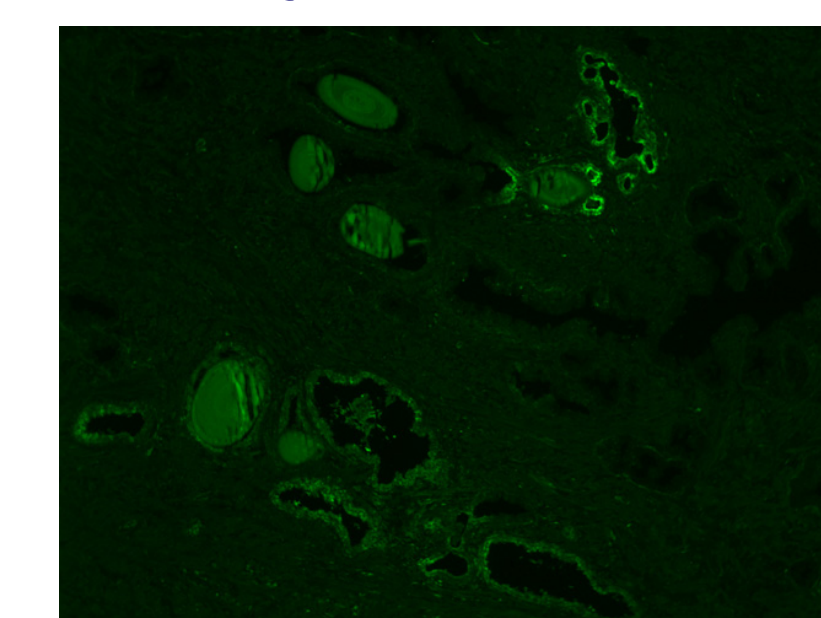
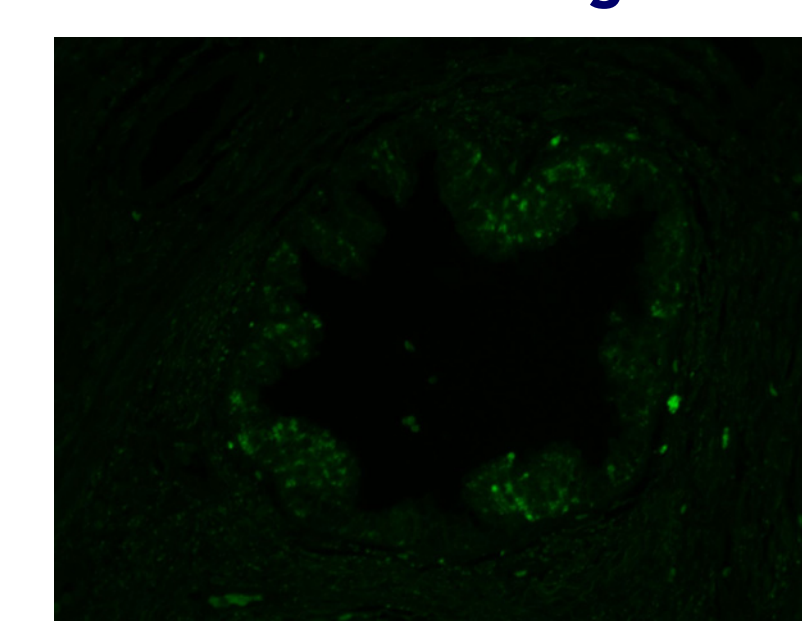


Figure 8.

No Primary Ab Controls

FITC-GAR Background



FL-dPEG[®]₁₂-GAR Background

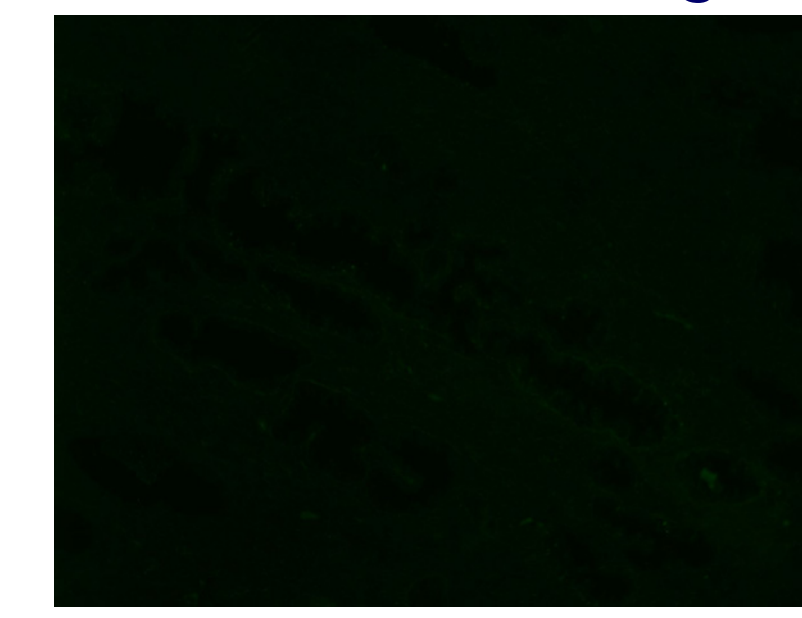


Figure 9. IHC Assays with Fixed MCF-7 Cells

- ◆ IHC experiments were performed with formaldehyde fixed MCF-7 Cells
 - The primary antibody was a mouse monoclonal against a golgi protein (GM130)
 - The secondary antibody was either FL-dPEG[®]₁₂-GAM or FITC-GAM at the same concentration
 - The incubation buffer was PBS/1% BSA/5% Goat Serum/0.05% Tween 20
- ◆ The green images (left side of slides) were taken using a 488 nm laser to excite fluorescein
- ◆ The green/red images (right side of slide) were taken using both the 488 nm laser and a 633 nm laser to excite the Draq5 dye used to stain the cell nuclei

Figure 10. FL-dPEG[®]₁₂-GAM

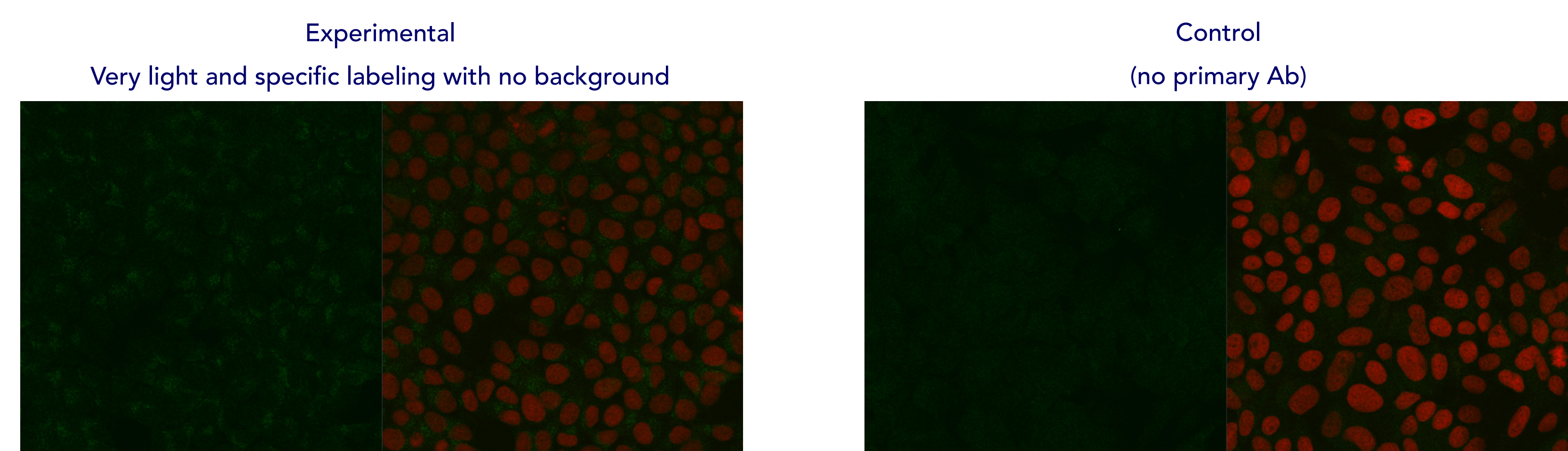


Figure 11. FITC-GAM R4

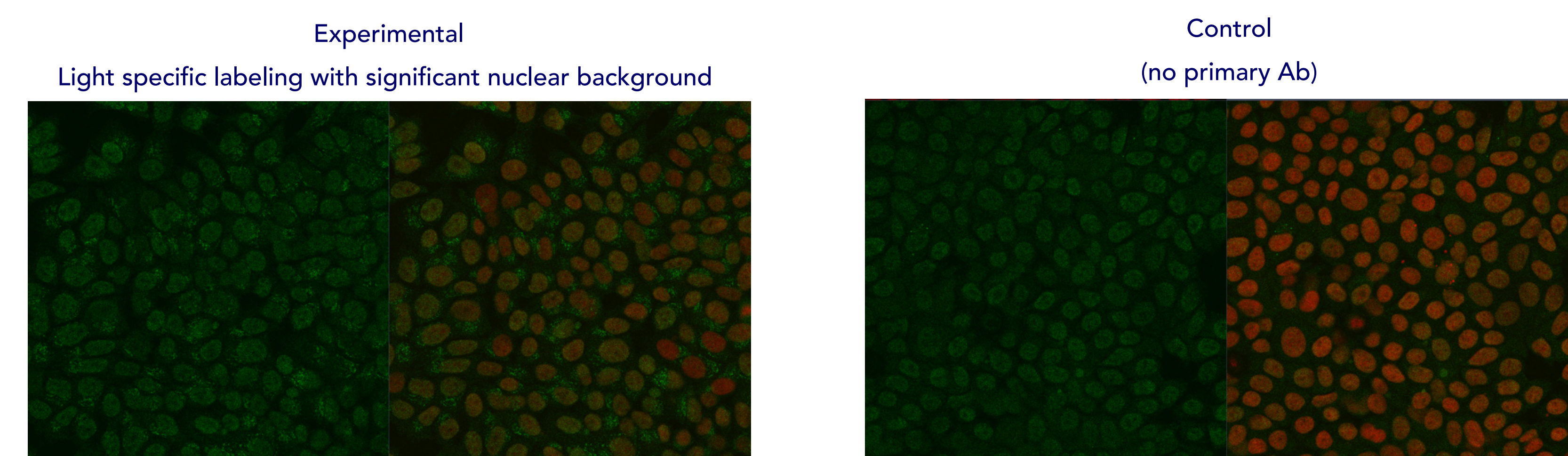


Figure 12.

Preparation of Additional Conjugates

- ◆ Fluorescein-dPEG[®]₁₂-Streptavidin (FL-dPEG[®]₁₂-SA)
 - Prepared via reaction of streptavidin with FL-dPEG[®]₁₂-NHS
 - FL-dPEG[®]₁₂-SA and Fluorescein-SA tested with MCF-7 cells using the mouse anti-golgi antibody and biotin-dPEG[®]₁₂-GAM
 - FL-dPEG[®]₁₂-SA showed specific staining and low backgrounds. The backgrounds were much higher for fluorescein-SA These results are similar to those found with FL-dPEG[®]₁₂-GAM.
- ◆ R-Phycoerythrin-dPEG[®]₂₄-SA
 - Prepared by oxime conjugation chemistry
 - Multiple products prepared with different molecular weight distributions
 - High activities via ELISA
 - Currently being tested in Luminex and Flow Cytometry applications

CONCLUSION

- ◆ The incorporation of a dPEG[®]₁₂ linker between fluorescein and an antibody allows at ~20 fluorescein molecules to be incorporated with little self quenching
- ◆ FL-dPEG[®]₁₂-GAM and FL-dPEG[®]₁₂-GAR bind specifically in IHC assays with much lower backgrounds than those obtained with the corresponding FITC labeled antibodies
- ◆ FL-dPEG[®]₁₂-SA shows less background staining than FITC-SA

Visit us at the American Qualex Booth #600

and

Dec. 5th 6:45pm-8:45pm, Tutorial Room 101

Novel Reagents: Increase Antibody and Conjugate Specificity, Sensitivity, Solubility; Reduce Nonspecific Binding