

Assaying protein conformational change in real time – A novel approach for target-based drug discovery

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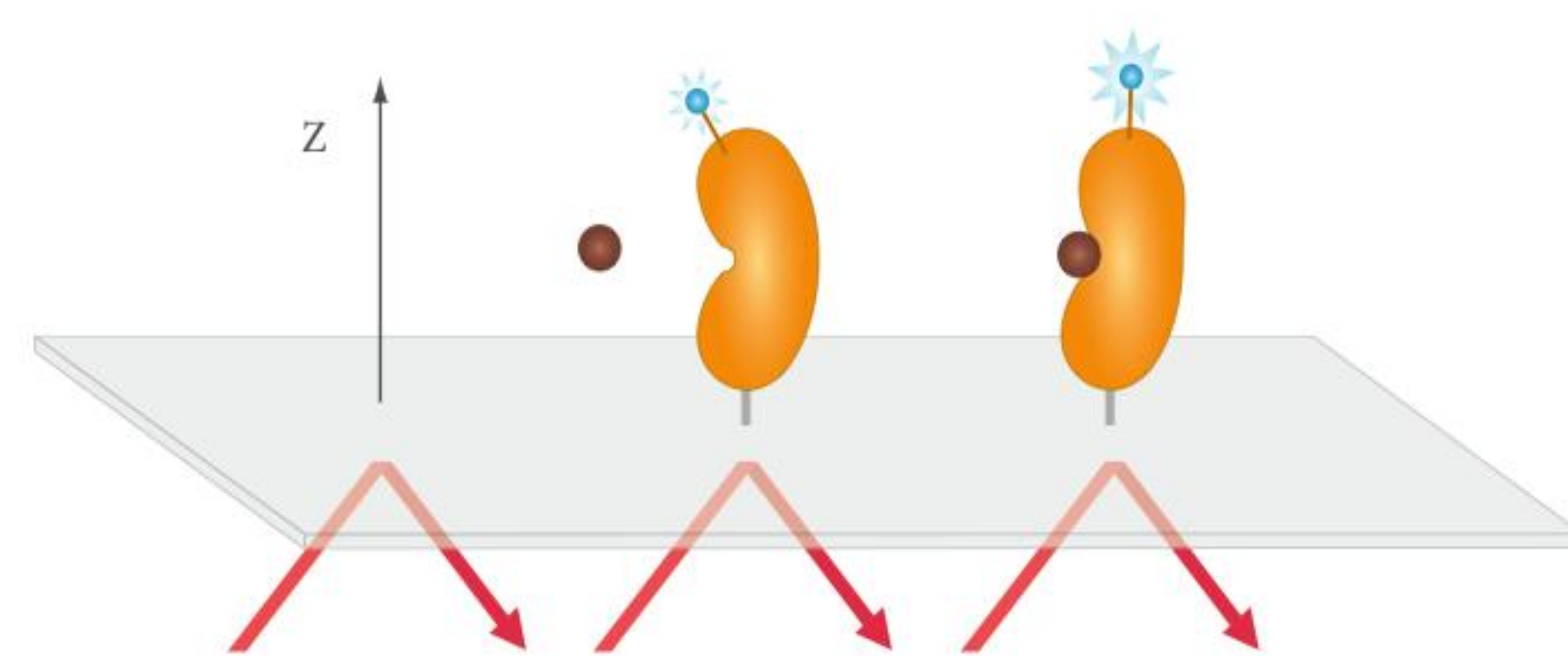
Abstract

Therapeutic targets that change conformation as part of the molecular mechanism of action constitute a growing, large fraction of recent first-in-class small-molecule therapeutics and have revolutionary advantages for treatment and blockbuster potential. For example, inhibitors of B-Raf bind to both active and inactive conformers of the target (Tsai et al., PNAS 2008). Here we describe the use of a novel optical technique, called Second Harmonic Generation (SHG), to develop highly informative assays for two important therapeutic targets known to undergo conformational changes upon ligand binding. SHG enables the monitoring of direct, real time conformational changes in proteins labeled with SHG-active dyes. SHG has a number of advantages above current methods for making conformational change measurements (e.g. X-ray crystallography), most notably that it is a dynamic, real-time biophysical measurement that does not require static crystals. In the current study, assays were performed on two blinded protein targets, which demonstrate the versatility of this approach, where information on the target is scarce. We report on assay design and methods that demonstrate the robust nature of the technique. Following random labeling of the target protein with an NHS ester of the SHG active dye, PyMPO, we examined a test panel of known positive and negative compounds, including known aggregators. We find that these SHG studies show complete agreement with ligand-induced conformational change observed in X-ray studies. The positive control compounds induce changes in SHG response, and these responses correlate to changes in secondary structure observed in X-ray crystallography studies. Furthermore, the IC₅₀ values obtained in a biochemical assay are in agreement with the values obtained from SHG analysis of a dilution series, and compound binding observed by SHG also correlates to results from SPR data for SHG-positive compounds. A known strong aggregator shows SHG response on both targets and is easily identifiable since the magnitude of the response is higher than those observed for positive compounds or the compound binds to unrelated targets in selectivity studies. Negative control compounds and a weak aggregator do not cause SHG responses. Overall, SHG assays hold the promise of significantly impacting the drug discovery process by generating data that directly reflect one of the most fundamental structural events in disease processes.

Introduction

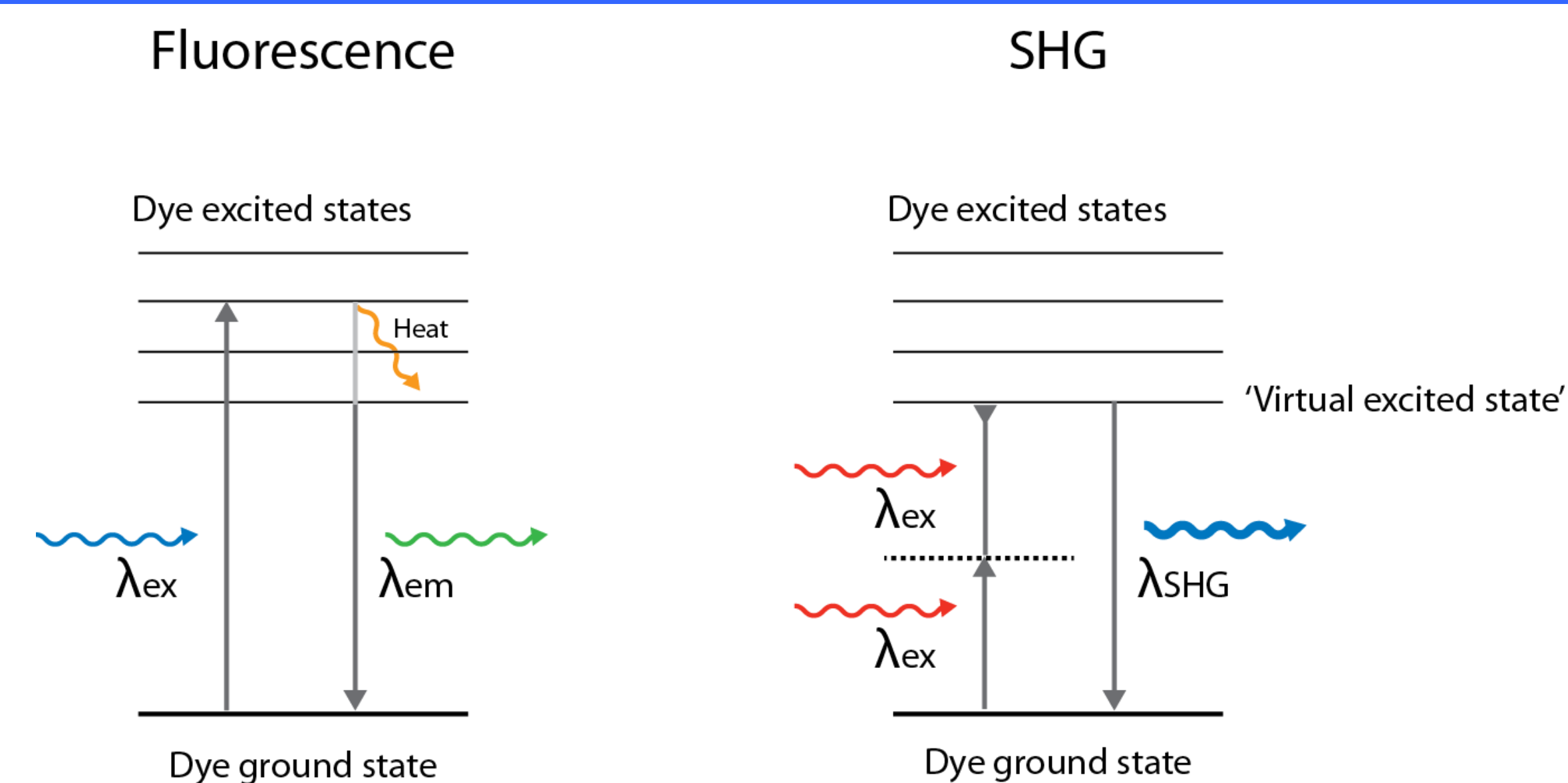
All proteins carry out their specific functions in living organisms by changing their conformation in some manner. One or more parts of the protein will bend or rotate to a subtle or large extent and this enables a binding or a physico-chemical reaction, such as catalysis, to occur. Identified therapeutics with a conformational molecular mechanism of action constitute a growing, large fraction of recent first-in-class small-molecule therapeutics and have revolutionary advantages for treatment and blockbuster potential. Here we describe the use of a novel optical technique, called Second Harmonic Generation (SHG), to develop a highly informative assay for an important therapeutic target. SHG enables the direct monitoring of direct, real time conformational change in proteins specifically labeled with SHG-active dyes. SHG has a number of advantages above current methods for making conformational change measurements (e.g. X-ray crystallography) most notably that it is a dynamic, real-time measurement and does not require static crystals. To evaluate this technology in the drug discovery process, SHG assays were performed on two blinded targets and compounds known to undergo structural changes upon ligand binding with a panel of compounds including positive controls, negative controls, and compounds known to aggregate in solution. We compared results from SHG studies with results from biochemical assays and SPR analyses.

Detecting Conformational Change by SHG



- Incident red light strikes the surface and through total internal reflection creates an evanescent wave polarized normal (Z-direction) to the plane of the surface and traveling just a short distance from the surface.
- After capture of labeled protein on a proprietary surface, baseline signal is generated by illuminating the biosensor. This signal is dependent on the position of the dye label relative to Z.
- Conformational change upon ligand binding changes the orientation of the dye relative to Z, resulting in a response.

SHG is Distinct from Fluorescence



- SHG is a distinct physical process, where two photons at lower wavelength are converted into one photon at higher wavelength
- Emission is highly sensitive to dye orientation relative to the Z-direction.

Methods

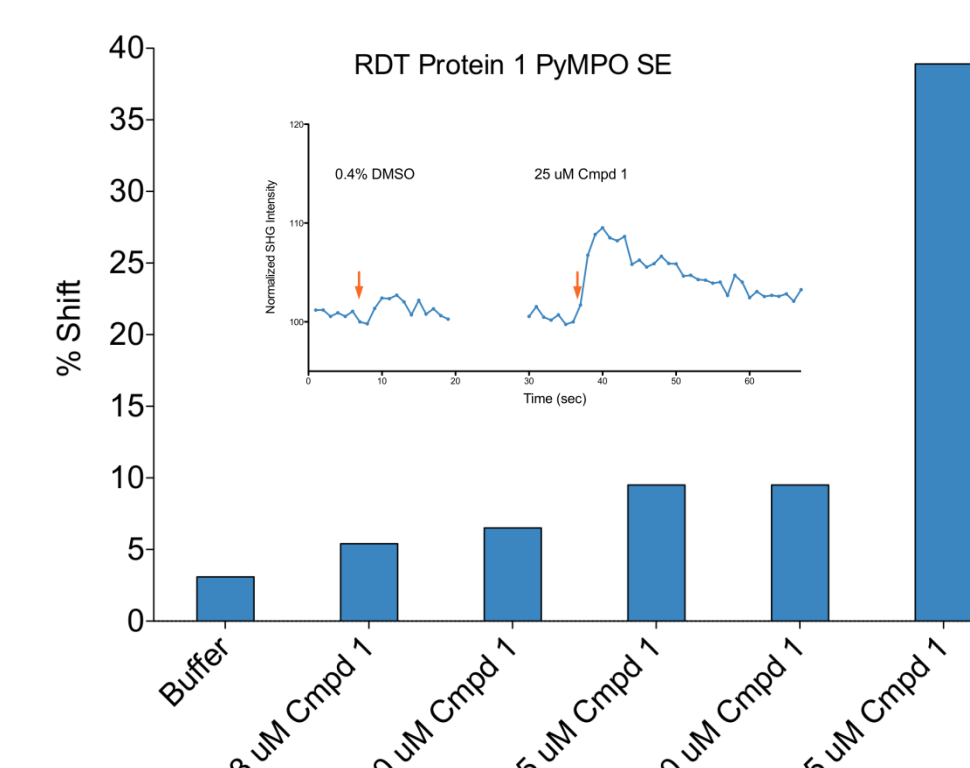
Labeling and SHG Analysis: Protein 1 was attached to a Biodesy slide via His-tag: Ni²⁺ interaction. Captured protein was labeled with PyMPO-NHS (Molecular Probes) at a ratio of 20:1 dye:protein in bicarbonate buffer and dye was removed by washing with assay buffer. Protein 2 was labeled prior to capture, and excess dye was removed with a G-50 spin column. The dye to protein molar ratio was 0.6. For SHG analysis, this protein was captured onto a Biodesy slide as described above, and SHG responses were monitored in the absence and presence of compound.

Study design: Blinded targets and compounds were provided by Roche, and all SHG analysis was performed by Biodesy. Other biophysical analyses including X-ray, SPR, and biochemical assays were conducted by Roche. For each protein, one positive control and one negative control were revealed for assay development. Additional compounds are known binders, negative controls, and compounds known to bind to proteins through non-specific mechanisms (Seidler et al. J Med Chem 2003 p4477), but were not revealed to Biodesy until after completion of the study.

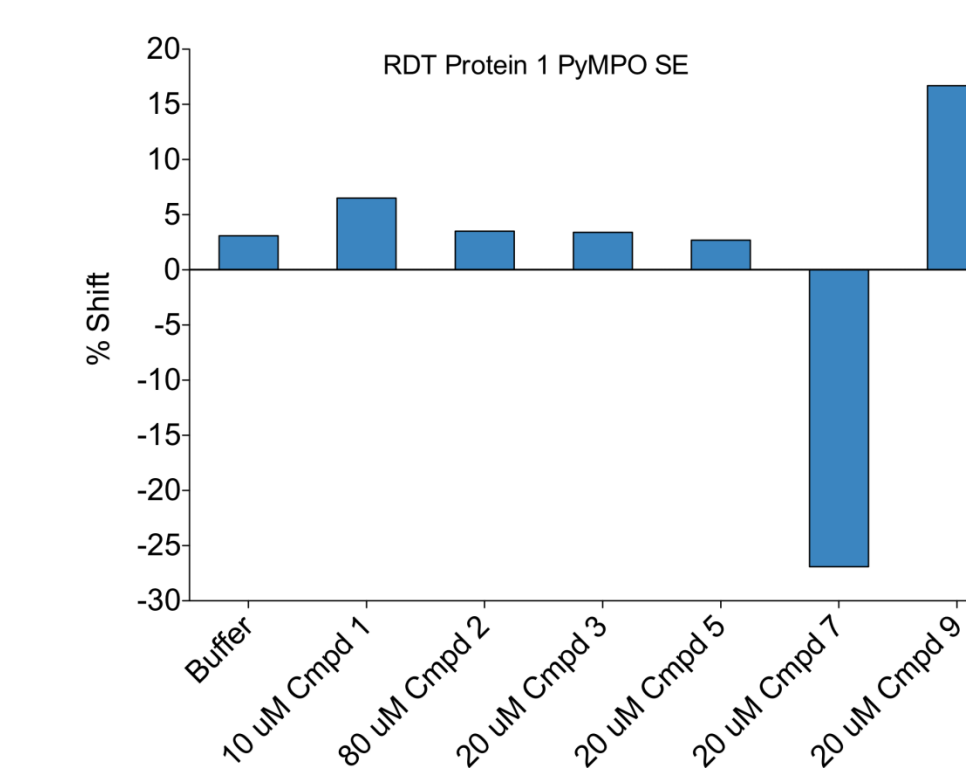
Results

SHG Analysis of Protein 1

SHG Responses for (+) Control



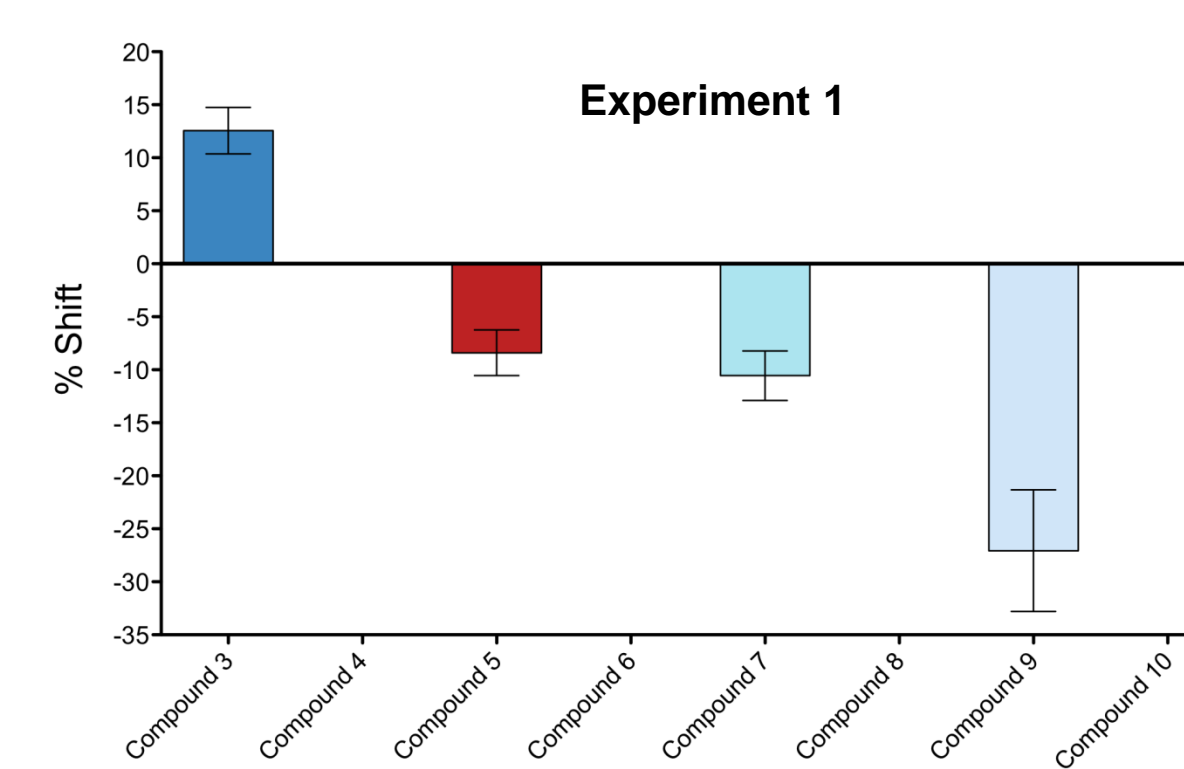
SHG Responses for (-) Controls



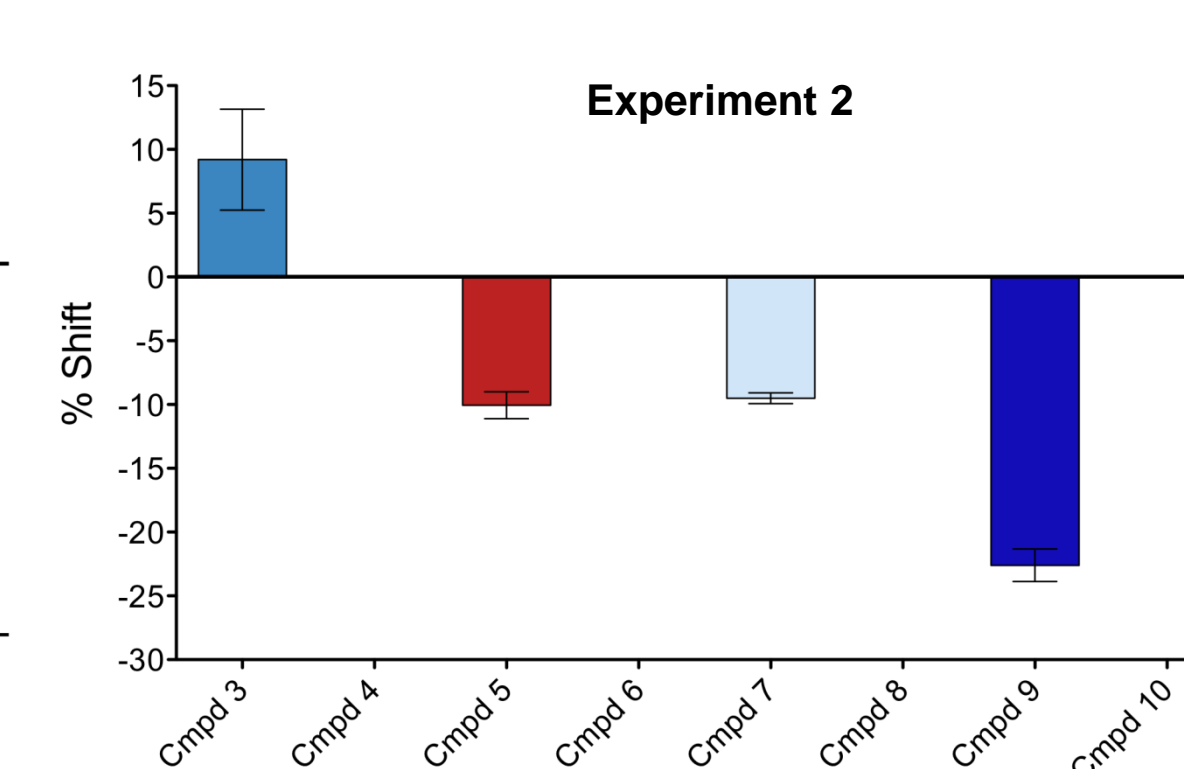
- This target was labeled non-specifically with SHG dye after immobilization at 5 μM on the biosensor.
- SHG response is observed for the positive control compound at 8-75 μM.
- Negative controls 2-5 do not show SHG response.
- Compound 7 binds to proteins non-specifically, and compound 9 is a strong aggregator. These compounds do not interact with Abl kinase which was labeled at cysteine residues with PyMPO-maleimide.

SHG Analysis of Protein 2

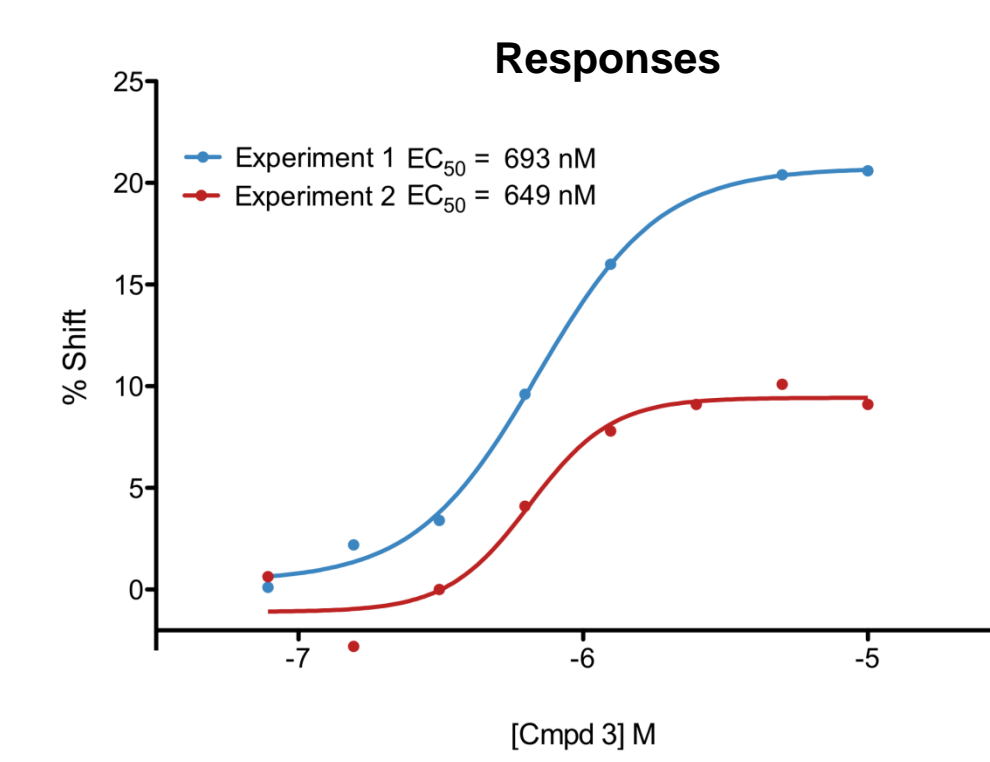
SHG Responses for a Test Panel of Eight Compounds



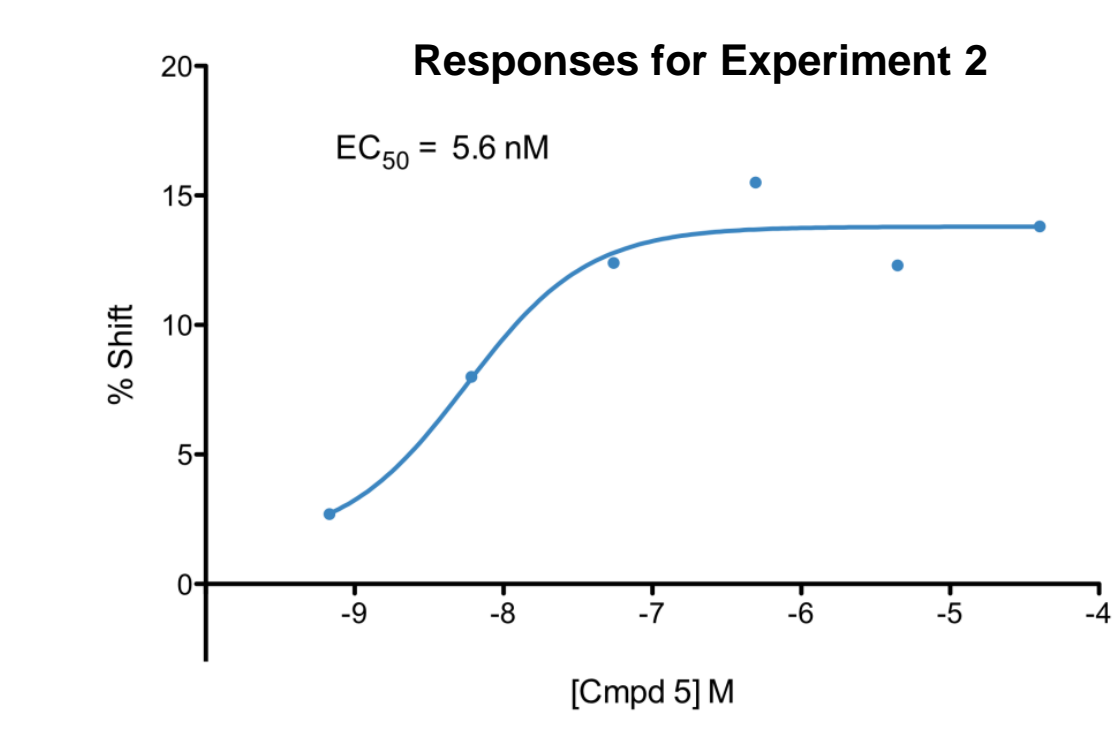
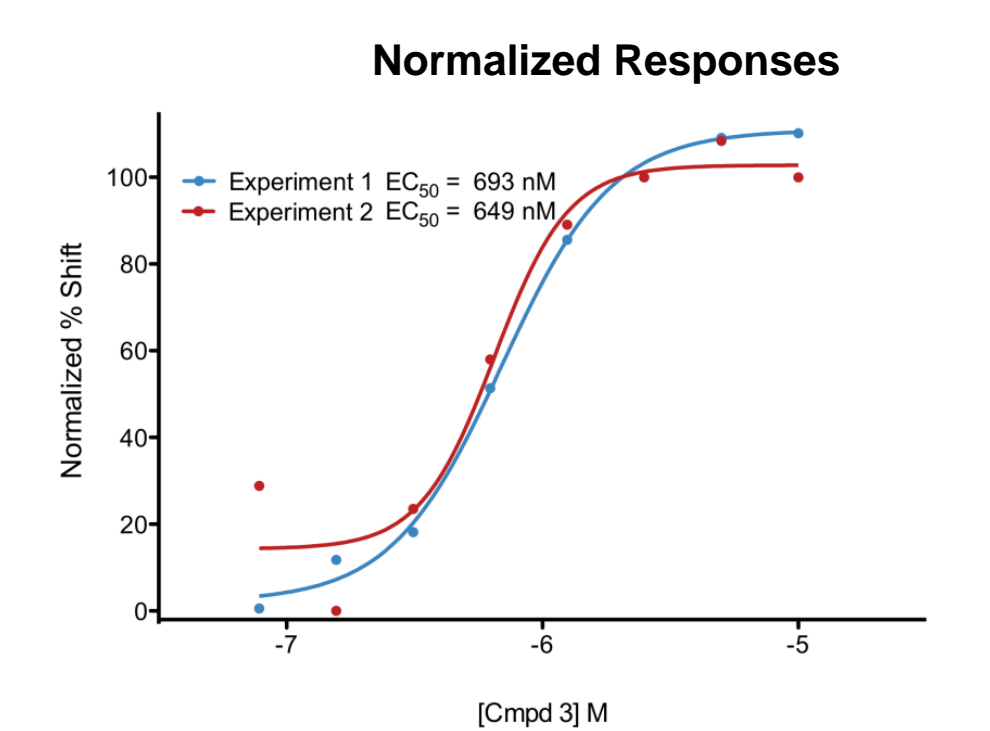
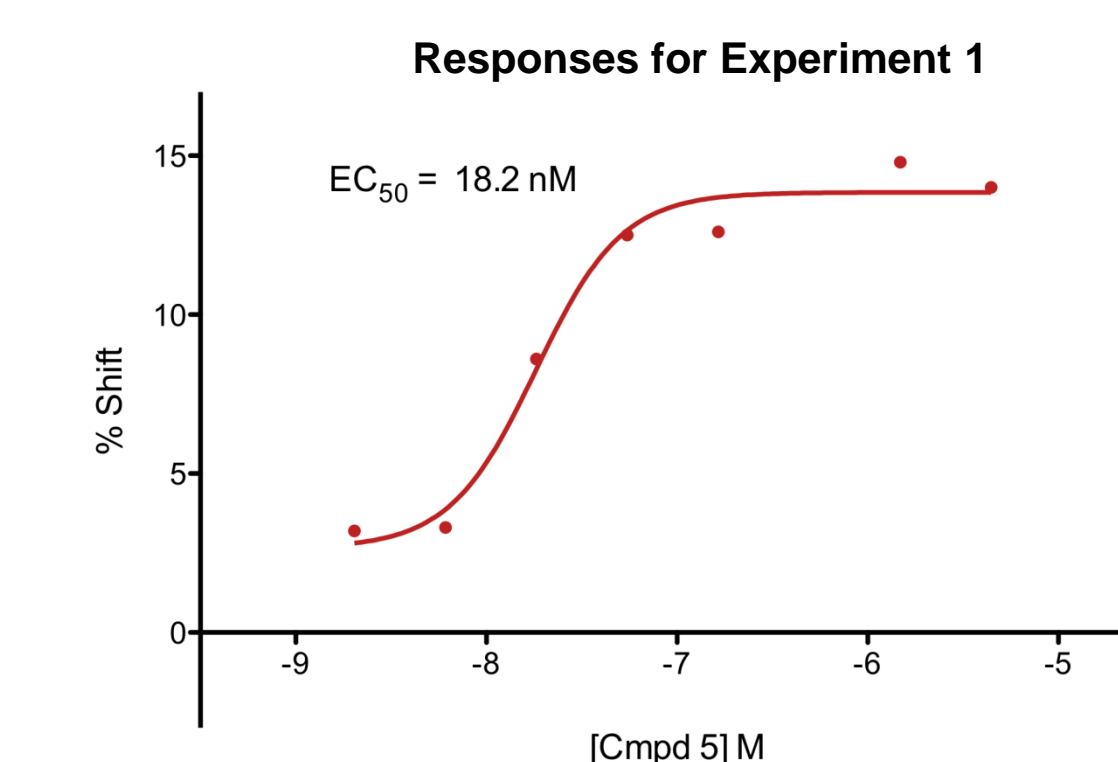
Reproducibility



SHG Determination of EC₅₀ for Compound 3



SHG Determination of EC₅₀ for Compound 5



Comparison of Results from SHG, SPR, Biochemical Assay, and X-ray analysis

Compound	Target	SHG Result	Biacore SPR Result	Biochemical IC ₅₀ Result	X-ray Structure Result
3	2	670nM	Binds to Target	950nM	Change of 0.5-2 Å in conformation in active site loop observed upon ligand binding
4	2	No response	Binds to Target	30nM	No change in conformation observed
5	2	12nM	Binds to Target	63nM	Change of 0.5-2 Å in conformation in active site loop observed upon ligand binding

Summary

- Proteins were successfully labeled with PyMPO-NHS, which labels lysines randomly at various locations on the protein with a stoichiometry of ~1 dye molecule per protein
- Known hits were confirmed for both targets
- Known inactive compounds were correctly identified
- A known strong aggregator and a known non-specific binder show SHG response on both targets, but not on a control protein. For target 2, a weak aggregator, a promiscuous fragment, and a non-binding compound do not generate SHG response.
- Preliminary potencies of "hits" were derived from response data and these results match data from a biochemical assay.
- SHG results are consistent with X-ray and SPR results.

Compounds

Compound	Compound Information
1	(+) control Protein 1
2	(-) control Protein 1
3	(+) control Protein 2
4	(-) control Protein 2
5	Unknown (binds Protein 2)
6	Unknown (promiscuous fragment)
7	Unknown (non-specific binder)
8	Unknown (non-binder)
9	Unknown (strong aggregator)
10	Unknown (weak aggregator)

References

- "Second-Harmonic Generation for Studying Structural Motion of Biological Molecules in Real Time and Space" Joshua S Salafsky, *Phys Chem Chem Phys* 2007, 9, 5704-5711
- "Detection of Protein Conformational Change by Optical Second-Harmonic Generation" Joshua S Salafsky, *J Chem Phys* 2006, 125, 74701
- "Promiscuous Aggregate-based Inhibitors Promote Enzyme Unfolding" Kristen E D Coan et al., *J Med Chem* 2009, 2067-2075
- "Identification and Prediction of Promiscuous Aggregating Inhibitors among Known Drugs" James Seidler et al., 2003, 46, 4477-4486