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Peer Review

Review of: "Optimizing Multifunctional Fluorescent Ligands for Intracellular Labeling"

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In the manuscript ''Optimizing Multifunctional Fluorescent Ligands for Intracellular Labeling'', the authors have presented an innovative concept in designing multifunctional ligands, containing fluorophores and affinity tags or pharmacological agents to label proteins covalently in living systems. As covalent labeling is an important tool in cell biology that allows manipulation and visualization of proteins in living cells and has proven useful in the elucidation of numerous biological processes. The manuscript is written excellently in broad terms, showing the design strategy and demonstrating it with experiments and results step by step, with well-validated and high-quality supporting data, and with impressive execution. Multifunctional fluorescent ligands turn into a single compound yielding relatively large molecules, which pose cell-permeability issues, and it was overcome by tuning the properties of modular rhodamine.

Authors have performed enormous work to show this proof of concept and reported it in different stages. First, they measured the distribution coefficients (logD7.4) of free rhodamine dyes and their HaloTag ligands and established a correlation with lactone–zwitterion equilibrium constants (KL–Z) in order to create cell-permeable multifunctional ligands. And then, they studied the effects of additional functionality on the dye structure in terms of equilibrium constants (KL–Z) and distribution coefficients (logD7.4), and it was observed that the changes are relatively minor; the parent dye exerts a strong effect on the resulting multifunctional ligand properties.

In the next step, they prepared a series of multifunctional ligands containing biotin, rhodamine, and HaloTag ligands, and their properties were investigated in vitro and in vivo. They successfully demonstrated the application of biotin-containing multifunctional ligands for affinity purification.

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They have discovered that the existing biotin ligands are suboptimal for streptavidin-mediated affinity purification using the HaloTag system due to poor cell permeability or insufficient linker length. In contrast, inserting a Si-rhodamine unit between the polar biotin and HaloTag ligand balances cellular permeability and functionality; biotin ligands enter cells, efficiently label HaloTag proteins, enable visualization, and present a biotin moiety accessible for affinity capture.

In the final step, the authors have extended this approach in designing cell-permeable multifunctional fluorescent HaloTag ligands with a pharmacological agent to translocate the protein BRD4 from euchromatin to the nucleolus or heterochromatin. They also discovered that the translocation of BRD4 to constitutive heterochromatin in cells expressing HaloTag–HP1a fusion proteins can lead to apparent increases in transcriptional activity.

The key point of this whole strategy in designing multifunctional ligands is the detailed investigation on lactone–zwitterion equilibrium constants (KL–Z) and distribution coefficients (logD7.4). It was shown that dyes with low KL–Z values, and therefore high distribution coefficients, could be useful scaffolds for creating cell-permeable multifunctional fluorescent ligands. The concept will facilitate the designing of new multifunctional chemical tools for understanding cellular processes in living systems.

The manuscript needs very minor corrections in the text with punctuation, and there are some mismatches in the Supp. Info. as mentioned below:

1. In abstracts, in line 8, there is a typo "sconstant," which needs correction.
2. Page 4, para 2, line 5: the relatively short PEG2-chloroalkane substrate motif can be problematic when the rhodamine moiety is replaced with other functionalities such as affinity tags or pharmacological agents. Cite the reference if known.
3. Punctuations throughout the text need to be re-checked, such as on page 4, para 2, line 11; page 7, para 1, line 8.
4. Page 7, paras 1 & 2 should be in one set.
5. Page 7, para 2, line 6: (**Scheme 1**, **SI** Appendix) is confusing because **Scheme 1** is in the main text, not in **SI**. Maybe it is better to write it like this: (**Scheme 1** and **SI** Appendix) or some other way.
6. The format of the word **Neuro2a** is not consistent in the main text and **SI**. It should be in the same format.
7. The manuscript title format in the main text and **Supp. Info.** is not the same.
8. Author affiliation in the main text and **Supp. Info.** is different.

9. Affiliation addresses in the main text and **Supp. Info.** are written differently.
10. In **SI**, scheme **S5**, in the last step, there should be compound **11**, not **10**, in order to get compound $\mathbf{21}_{\mathrm{HTL}}$.

Declarations

Potential competing interests: No potential competing interests to declare.