Supplementary Material For

Site-Specific Dual Encoding and Labeling of Proteins via Genetic Code Expansion

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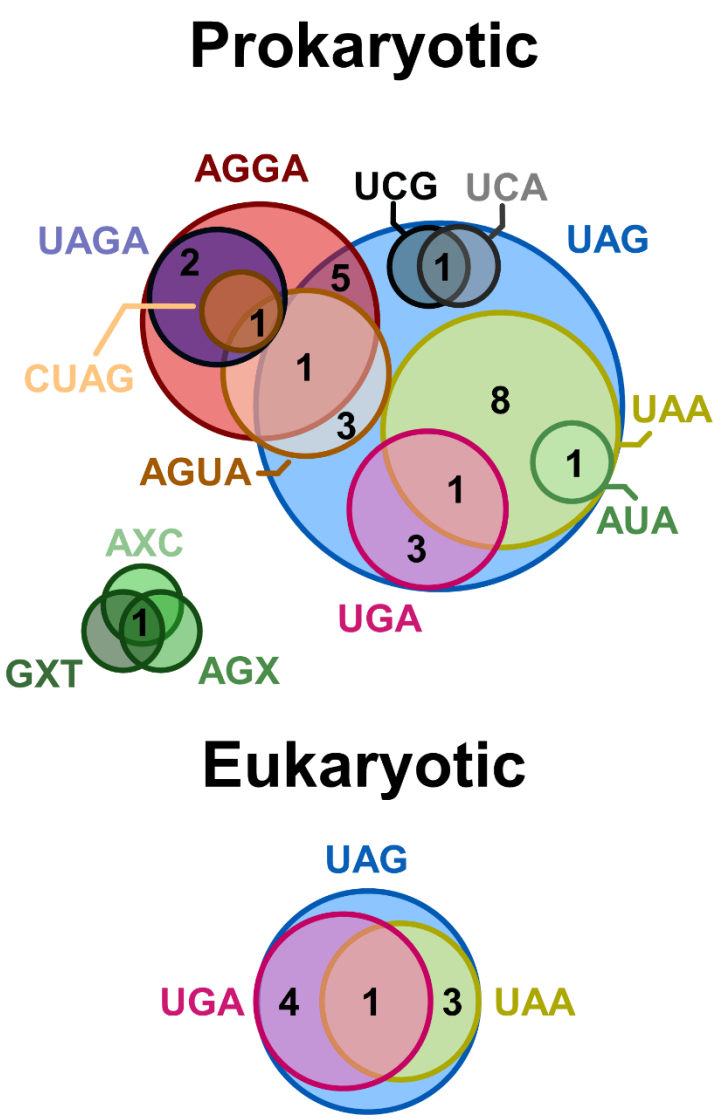


Figure S1.
Overview of blank codon combinations used in the Table 1 publications. Separate Venn diagrams are presented for the prokaryotic (above) and eukaryotic (below) systems studied. Different colored circles are used for each codon type with the size of the circles proportional to the number of times that codon has been used. The areas of circle overlap are not meaningful, and instead the number is given within each region of the diagram conveys the number of studies using that pair (or triplet or quadruplet) of codons. For the codons "AXC," "AGX," and "GXT", X refers to a noncanonical base present in the codon; these three codons were used in combination independently of other codons, and hence do not overlap any other codons other than one another.

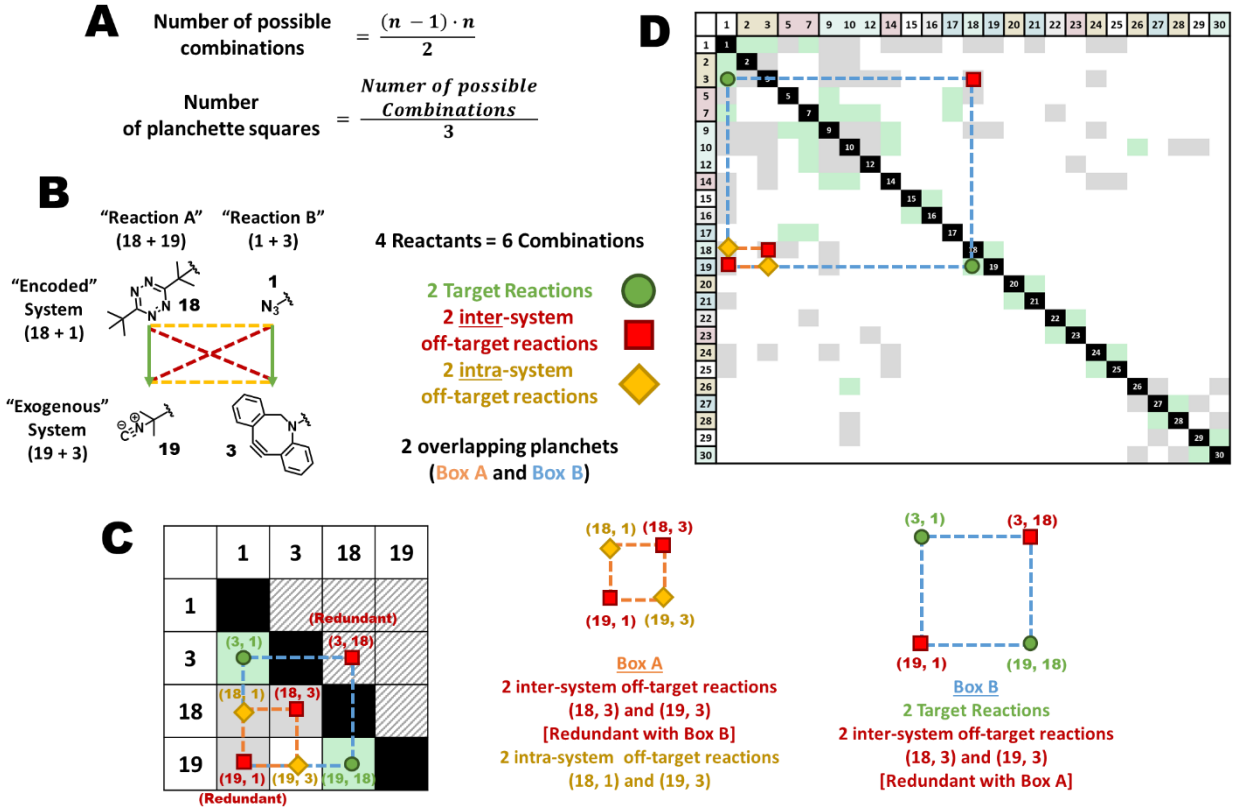


Figure S2.
Explanation on how the orthogonality matrix from Figure 6 can be used with planchettes for discovering new reactions that are useful for multiple (dual and higher) labeling studies. Compounds are as defined in Figure 6. **A** Equation for determining a reasonable number of planchette squares needed for a given number of reactants "*n*". Two reactants are needed per unique labeling reaction (e.g. 4 reactants for dual labeling and 6 reactants for triple labeling). Using "*n*" as the total number of reactants needed for the planned experiment, equations are given for calculating the possible reaction combinations (above) and the minimal number of overlapping planchettes needed to assess their mutual orthogonalities on one side of the matrix diagonal (a set of symmetrical planchettes can also be placed on the opposing side of the diagonal). **B** A dual labeling example using reactants **1**, **3**, **18**, and **19,** with target reactions A and B and the encoded and exogenous system reactants as illustrated on the top, and left sides, respectively. As shown on the right (using the style of Figure 4a), these four reactants can participate in 6 potential reactions: two intended target reactions; two "inter-system" off-target reactions; and two "intra-system" off-target reactions. **C** Defining the required planchettes for the example introduced in panel B. On the left is a simplified reactivity matrix (based on Figure 6) that only shows the four relevant reactants and reveals how two planchettes – one nested within the other – are sufficient to cover all 6 relevant reactant combinations. These two planchettes are shown separately to the right with the names Box A (with vertices covering the two inter-system off-target reactions and the two target reactions), and Box B (with vertices covering the two intra-system off-target reactions and the two inter-system off-target reactions). In this planchette arrangement, the inter-system off-target reactions are covered by both boxes, making them redundant. This is how two overlapping planchets, each with four vertices, and two redundant vertices are able cover six unique combinations. Note, different arrangements of the planchettes can be chosen, including some

where the redundant vertices may be intra-system off-target reaction combinations (as in Figure 6). **D** Positioning the two overlapping planchets from panel **C** on the Figure 6 orthogonality matrix. Note, for three chemically orthogonal reactions composed of six total reactants, there are 15 possible reactions, which can be covered by five overlapping boxes. Further examples of how this approach can be used to discover new groups of chemically orthogonal reactions, see Figure S11.

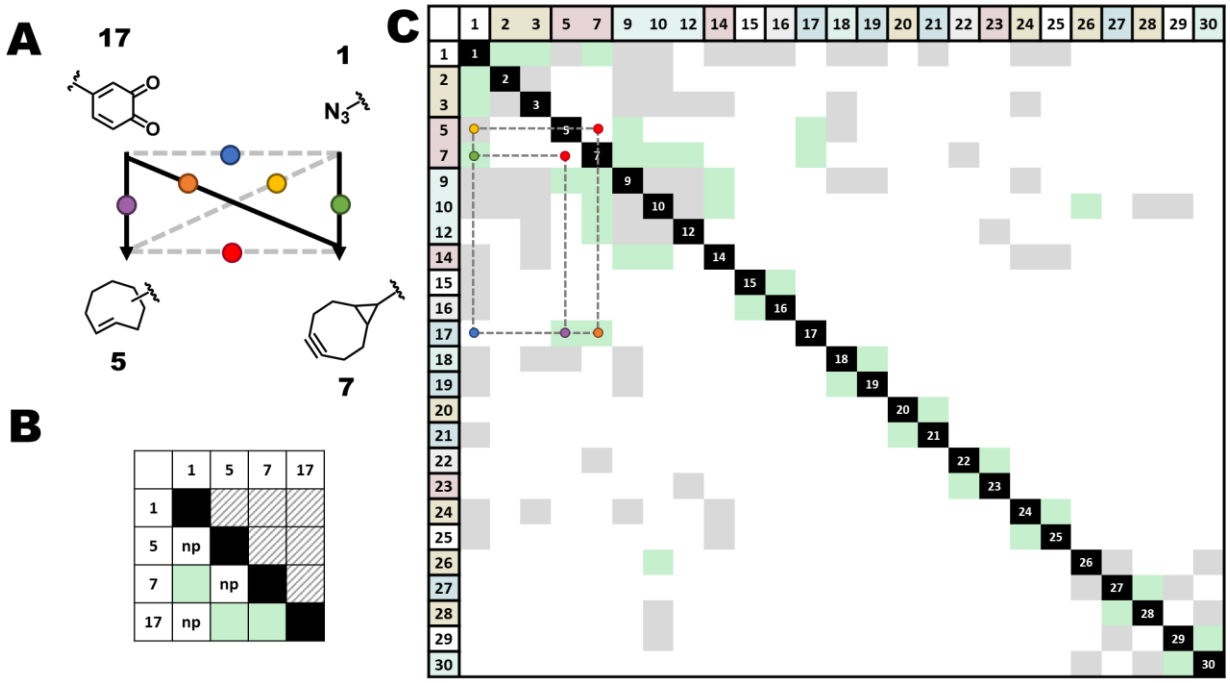


Figure S3.
Orthogonality matrix for reactions presented in Bruins et al., 2018[13].
A Reactivity relationships between all six combinations of four reactants. Solid black arrows indicate reactions that were empirically tested and observed to occur. Dashed light gray arrows indicate reactions that were not empirically tested. Each arrow is differentiated by a distinct colored circle. **B** Truncated reactivity matrix between all combinations of reactants. Green boxes indicated combinations that were empirically tested and observed to occur. White boxes with "np" indicate that reaction combination was not performed. Dashed boxes indicate redundant reaction combinations. **C** Full reactivity matrix of the combinations in the context of all other combinations with planchettes overlapped. Planchette vertices are color coded as in panel **A**. Note that for this combination, reactants **17** and **7** participate in an observed reaction; however, the possibility of cross-reactivity is avoided by order of additions.

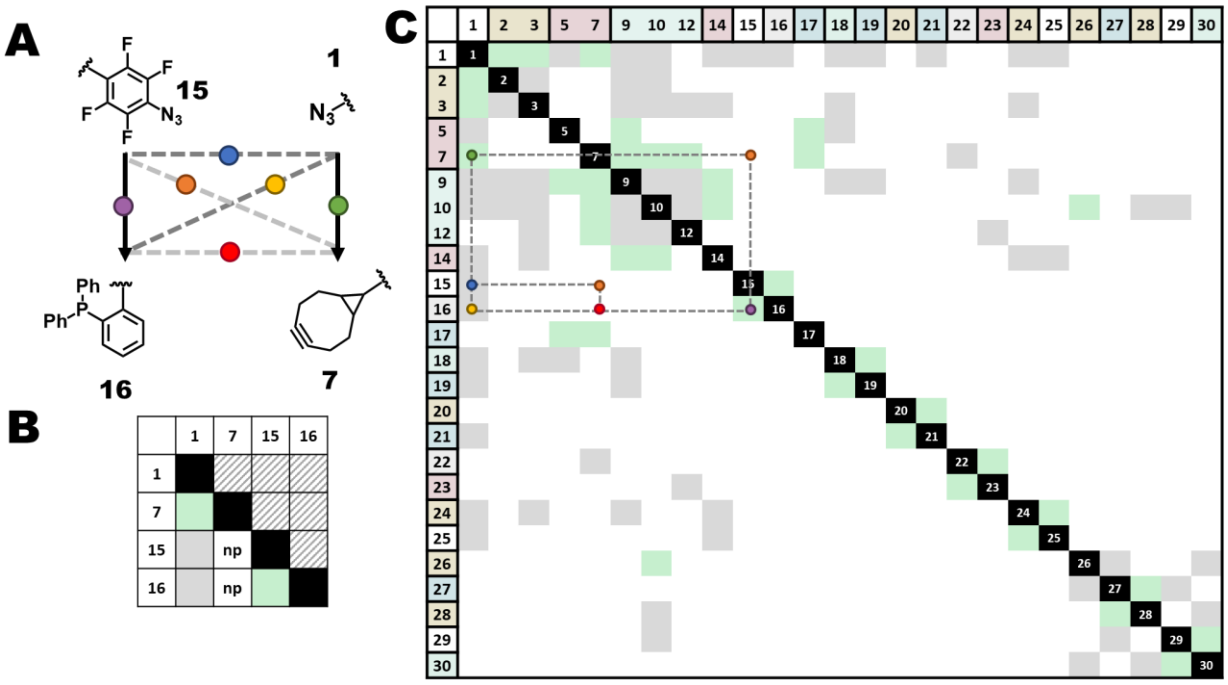


Figure S4.
Orthogonality matrix for reactions presented in Cheng et al., 2019[141].
A Reactivity relationships between all six combinations of four reactants. Solid black arrows indicate reactions that were empirically tested and observed to occur. Dashed dark gray arrows represent reactions that were empirically tested and observed to not occur. Dashed light gray arrows indicate reactions that were not empirically tested. Each arrow is differentiated by a distinct colored circle. **B** Truncated reactivity matrix between all combinations of reactants. Green boxes indicate combinations that were empirically tested and observed to occur. Gray boxes indicate combinations that were empirically tested and observed to not occur. White boxes with "np" indicate that reaction combination was not performed. Dashed boxes indicate redundant reaction combinations. **C** Full reactivity matrix of the combinations in the context of all other combinations with planchettes overlapped. Planchette vertices are color coded as in panel **A**. Note that for this example, moeities **15** and **1** are present on the same molecule, and are thus presumed to not react.

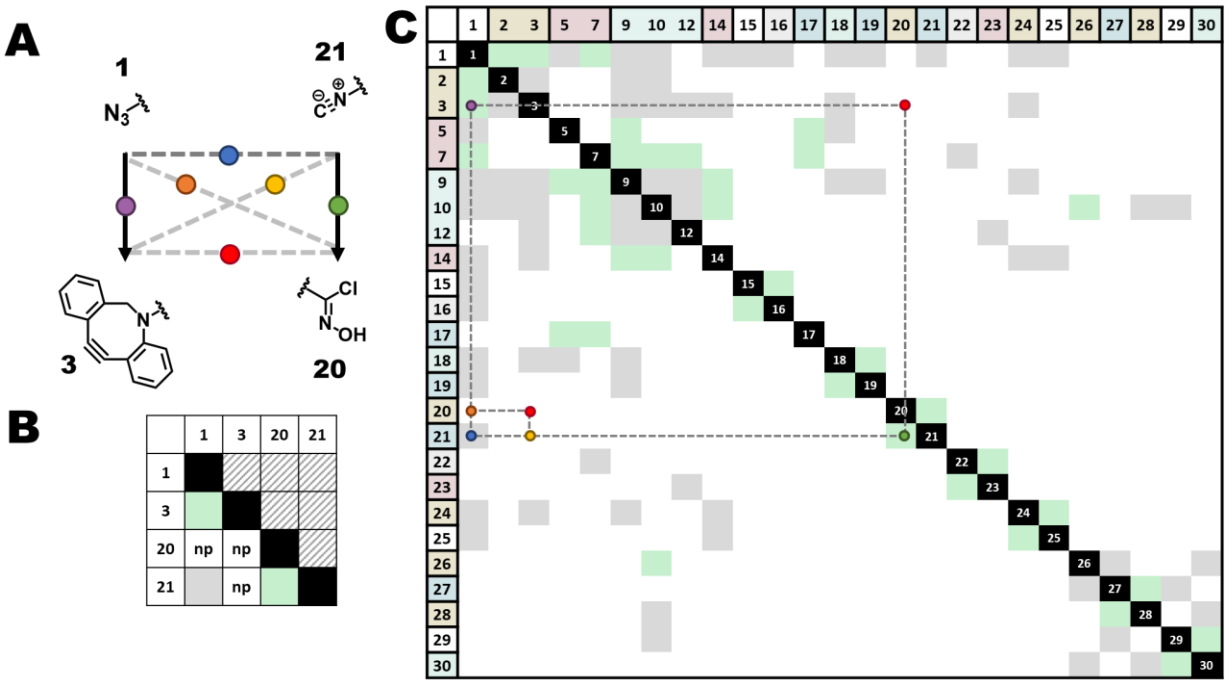
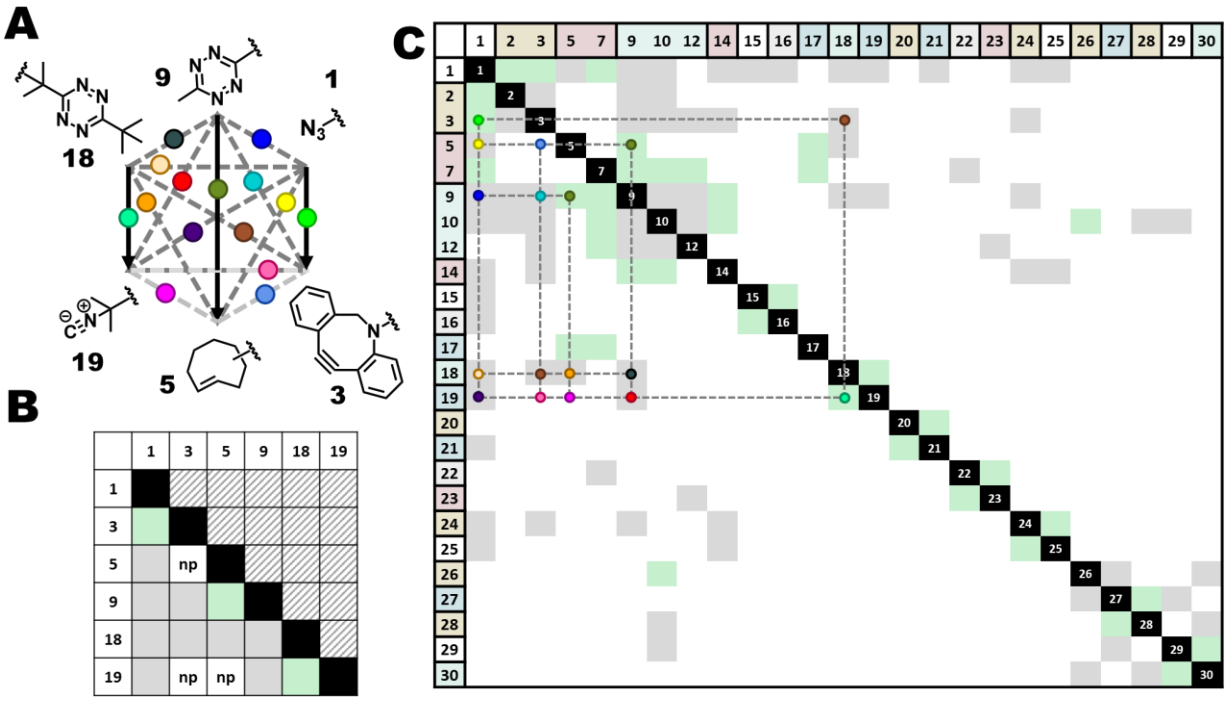


Figure S5.
Orthogonality matrix for reactions presented in Shäfer et al., 2019[142].
A Reactivity relationships between all six combinations of four reactants. Solid black arrows indicate reactions that were empirically tested and observed to occur. Dashed dark gray arrows represent reactions that were empirically tested and observed to not occur. Dashed light gray arrows indicate reactions that were not empirically tested. Each arrow is differentiated by a distinct colored circle. **B** Truncated reactivity matrix between all combinations of reactants. Green boxes indicate combinations that were empirically tested and observed to occur. Gray boxes indicate combinations that were empirically tested and observed to not occur. White boxes with "np" indicate that reaction combination was not performed. Dashed boxes indicate redundant reaction combinations. **C** Full reactivity matrix of the combinations in the context of all other combinations with planchettes overlapped. Planchette vertices are color coded as in panel **A**. Note that for this example, while not explicitly tested, handles **1** and **21** were metabolically encoded over a period of ~3 d and are presumed to not react.



	1	3	5	9	18	19
1						
3						
5		np				
9						
18						
19		np	np			

Figure S6.
Orthogonality matrix for reactions presented in Tu et al., 2019[144].
A Reactivity relationships between all six combinations of four reactants. Solid black arrows indicate reactions that were empirically tested and observed to occur. Dashed dark gray arrows represent reactions that were empirically tested and observed to not occur. Dashed light gray arrows indicate reactions that were not empirically tested. Each arrow is differentiated by a distinct colored circle. **B** Truncated reactivity matrix between all combinations of reactants. Green boxes indicate combinations that were empirically tested and observed to occur. Gray boxes indicate combinations that were empirically tested and observed to not occur. White boxes with "np" indicate that reaction combination was not performed. Dashed boxes indicate redundant reaction combinations. **C** Full reactivity matrix of the combinations in the context of all other combinations with planchettes overlapped. Planchette vertices are color coded as in panel **A**.

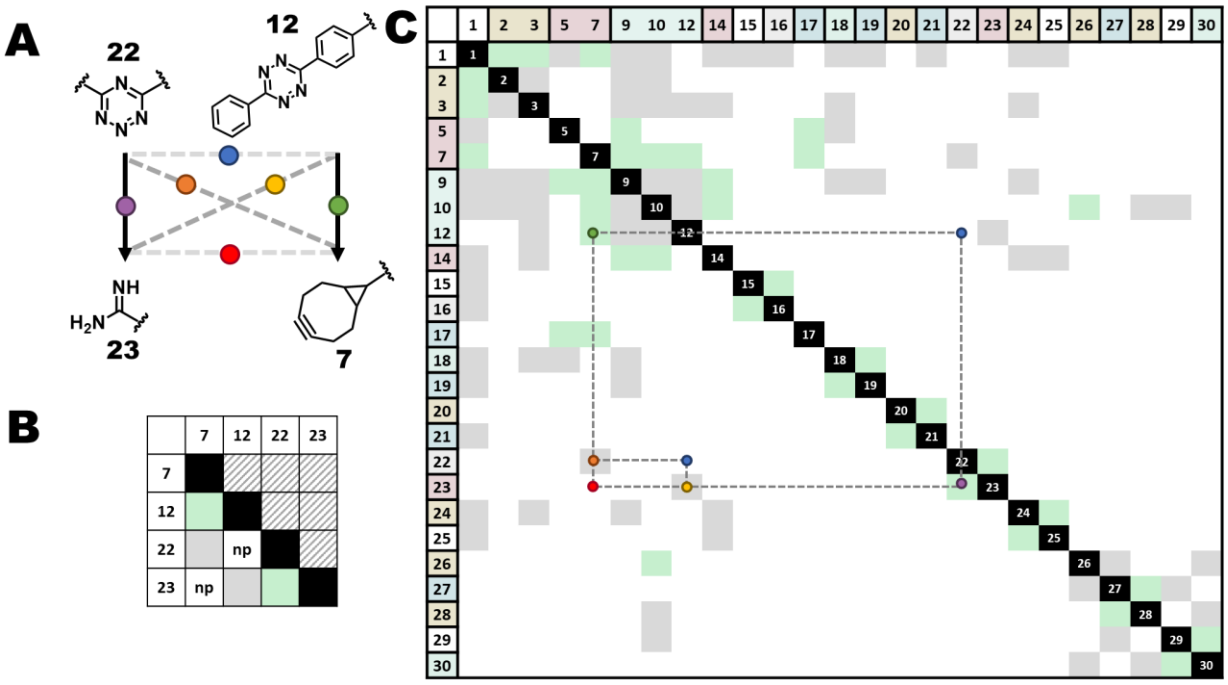


Figure S7.
Orthogonality matrix for reactions presented in Wu and Boger, 2019[145].
A Reactivity relationships between all six combinations of four reactants. Solid black arrows indicate reactions that were empirically tested and observed to occur. Dashed dark gray arrows represent reactions that were empirically tested and observed to not occur. Dashed light gray arrows indicate reactions that were not empirically tested. Each arrow is differentiated by a distinct colored circle. **B** Truncated reactivity matrix between all combinations of reactants. Green boxes indicate combinations that were empirically tested and observed to occur. Gray boxes indicate combinations that were empirically tested and observed to not occur. White boxes with "np" indicate that reaction combination was not performed. Dashed boxes indicate redundant reaction combinations. **C** Full reactivity matrix of the combinations in the context of all other combinations with planchettes overlapped. Planchette vertices are color coded as in panel **A**.

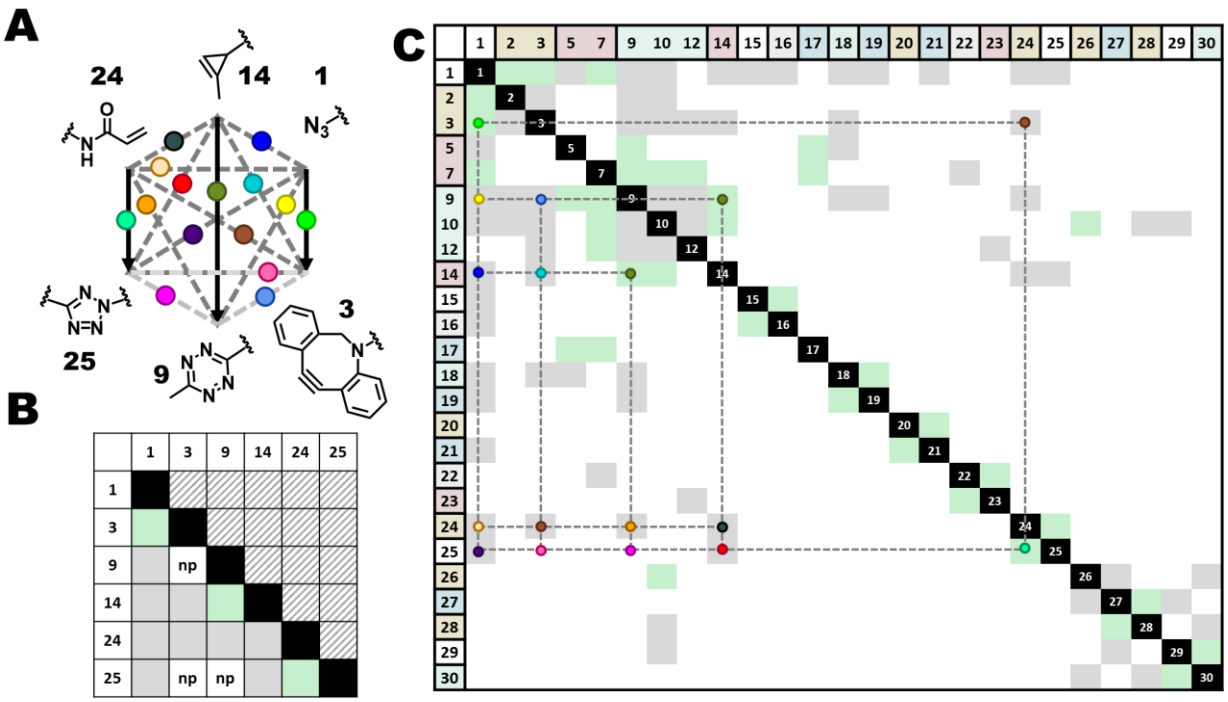
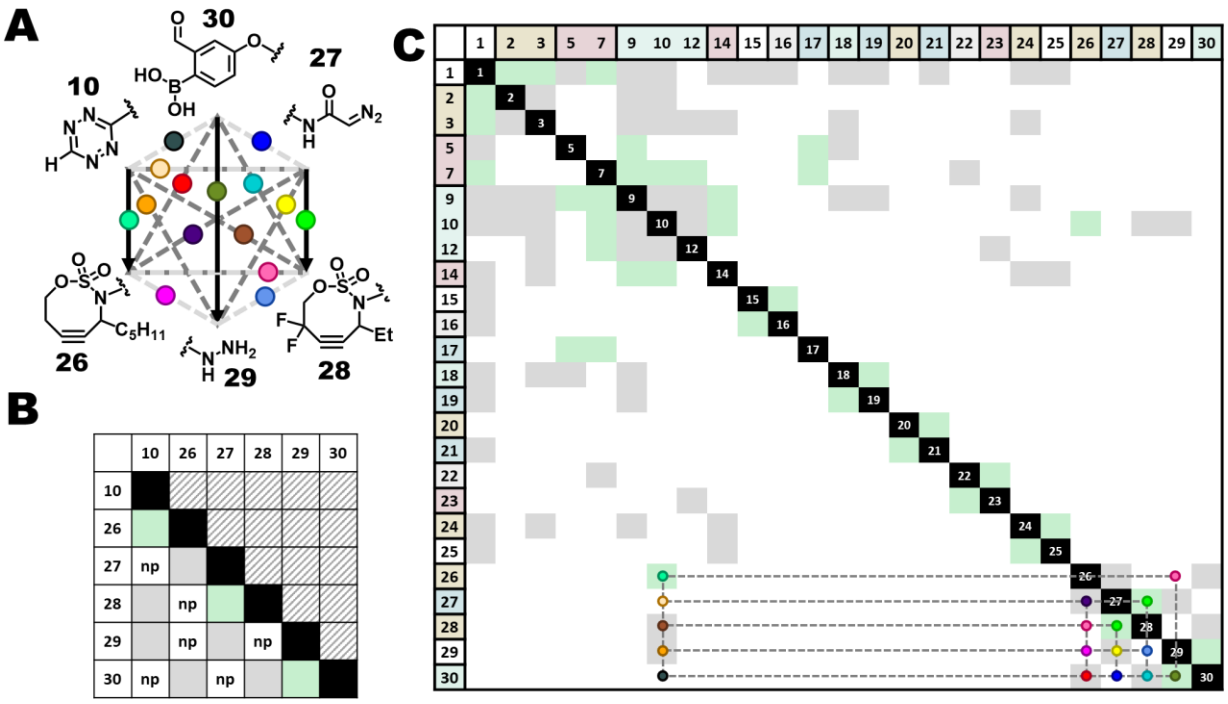


Figure S8.
Orthogonality matrix for reactions presented in Schart et al., 2019[146].
A Reactivity relationships between all six combinations of four reactants. Solid black arrows indicate reactions that were empirically tested and observed to occur. Dashed dark gray arrows represent reactions that were empirically tested and observed to not occur. Dashed light gray arrows indicate reactions that were not empirically tested. Each arrow is differentiated by a distinct colored circle. **B** Truncated reactivity matrix between all combinations of reactants. Green boxes indicate combinations that were empirically tested and observed to occur. Gray boxes indicate combinations that were empirically tested and observed to not occur. White boxes with "np" indicate that reaction combination was not performed. Dashed boxes indicate redundant reaction combinations. **C** Full reactivity matrix of the combinations in the context of all other combinations with planchettes overlapped. Planchette vertices are color coded as in panel **A**.



	10	26	27	28	29	30
10						
26						
27	np					
28		np				
29		np		np		
30	np		np			

Figure S9.
Orthogonality matrix for reactions presented in Hu et al., 2020[127].
A Reactivity relationships between all six combinations of four reactants. Solid black arrows indicate reactions that were empirically tested and observed to occur. Dashed dark gray arrows represent reactions that were empirically tested and observed to not occur. Dashed light gray arrows indicate reactions that were not empirically tested. Each arrow is differentiated by a distinct colored circle. **B** Truncated reactivity matrix between all combinations of reactants. Green boxes indicate combinations that were empirically tested and observed to occur. Gray boxes indicate combinations that were empirically tested and observed to not occur. White boxes with "np" indicate that reaction combination was not performed. Dashed boxes indicate redundant reaction combinations. **C** Full reactivity matrix of the combinations in the context of all other combinations with planchettes overlapped. Planchette vertices are color coded as in panel **A**.

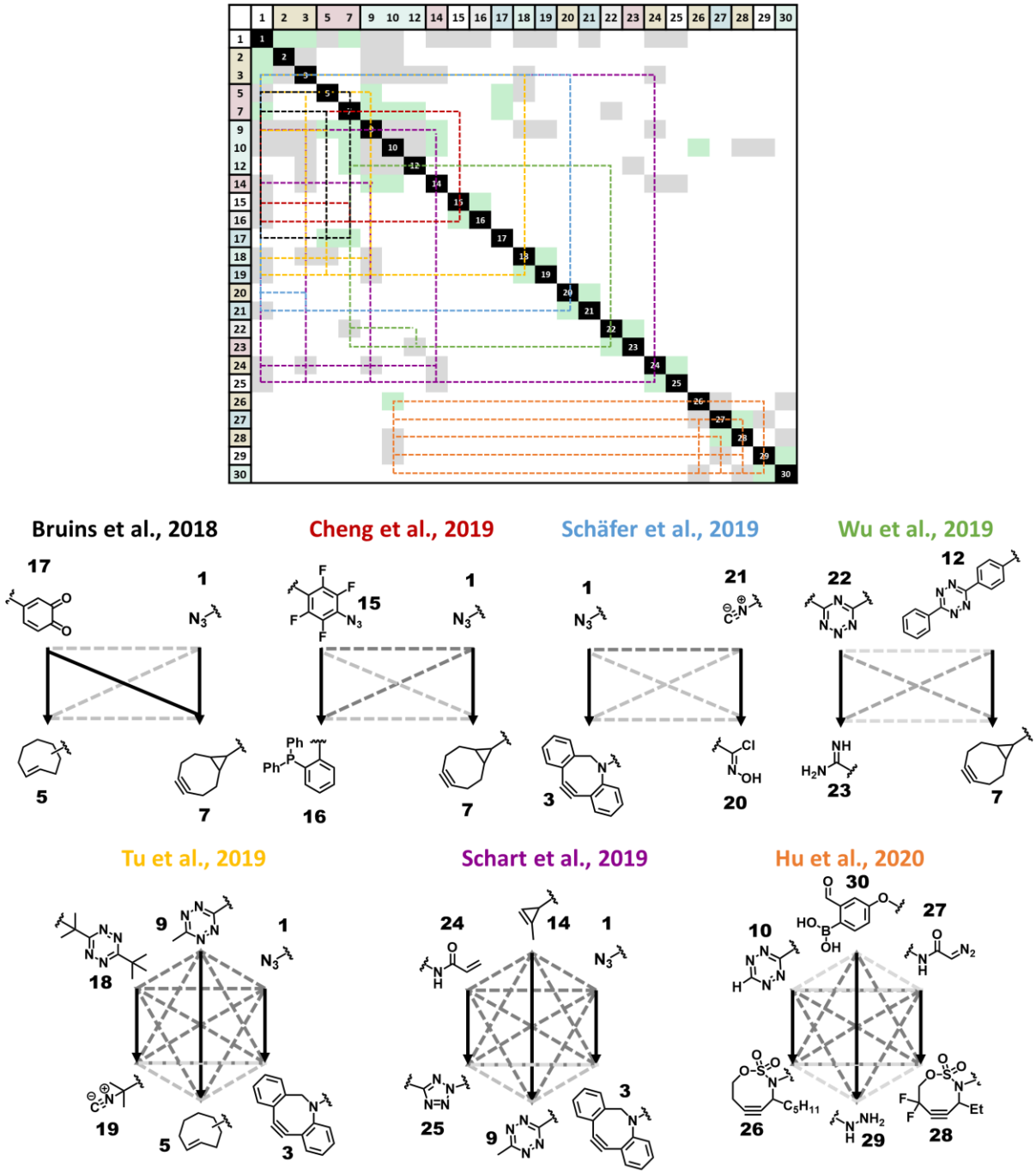


Figure S10.
Orthogonality matrix for all examples discussed in this review including all overlapping planchettes (from Figures S3 - S9). Planchettes are color coded according to the identifying reference text for each reaction combination shown below.

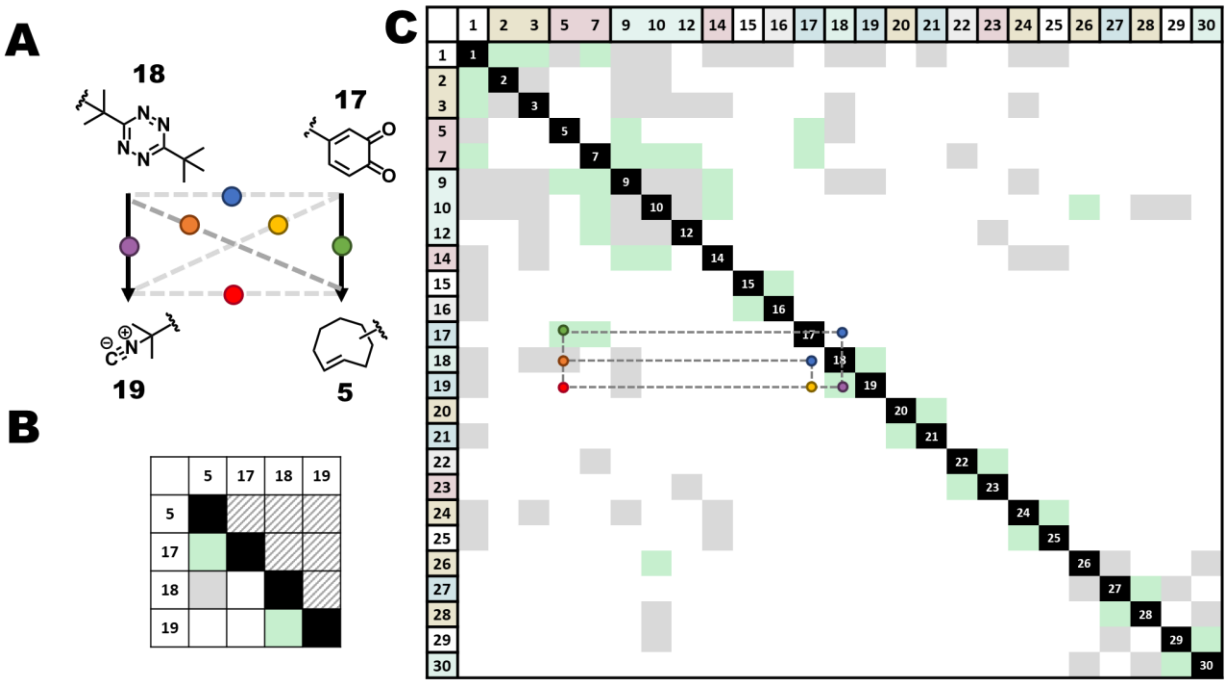


Figure S11.
Example of a new reaction pair combination, consisting of reactants **15**, **17**, **18**, and **19**, discovered from the orthogonality table present in Figure 6. In this example, handles **18** and **17** could be encoded into the same protein and subsequently orthogonally labeled. Since the reaction between **18** and **5** has been empirically observed to not appreciably occur, the reaction between **17** and **5** could be performed first, followed by the reaction between **18** and **19** via order of additions (this may not be necessary though, if the reaction between **17** and **19** is found to not substantially occur). **A** Reactivity relationships between all six combinations of four reactants. Solid black arrows indicate reactions that were empirically tested and observed to occur. Dashed dark gray arrows represent reactions that were empirically tested and observed to not occur. Dashed light gray arrows indicate reactions that were not empirically tested. Each arrow is differentiated by a distinct colored circle. **B** Truncated reactivity matrix between all combinations of reactants. Green boxes indicate combinations that were empirically tested and observed to occur. Gray boxes indicate combinations that were empirically tested and observed to not occur. White boxes with "np" indicate that reaction combination was not performed. Dashed boxes indicate redundant reaction combinations. **C** Full reactivity matrix of the combinations in the context of all other combinations with planchettes overlapped. Planchette vertices are color coded as in panel **A**.

References

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