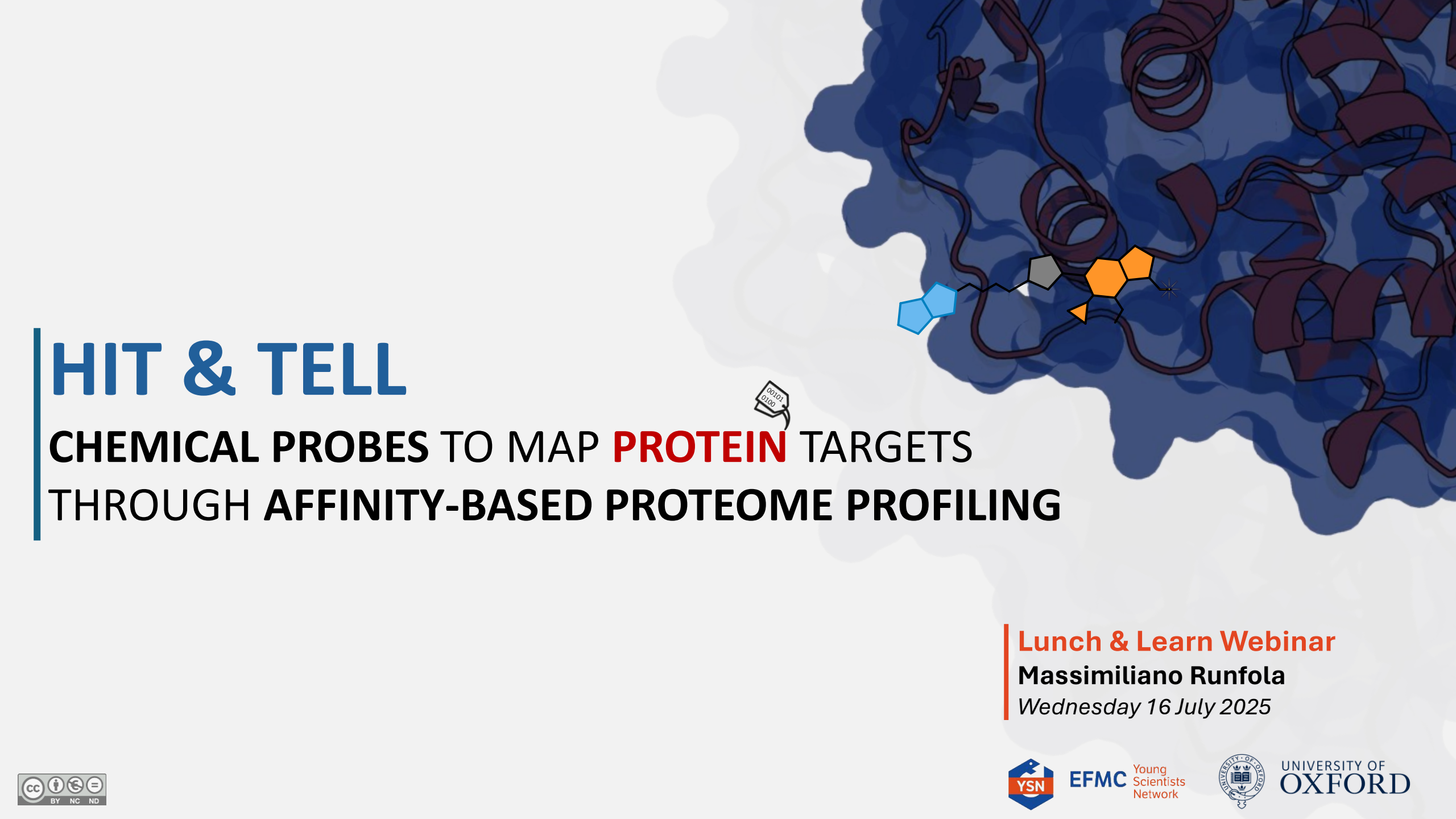




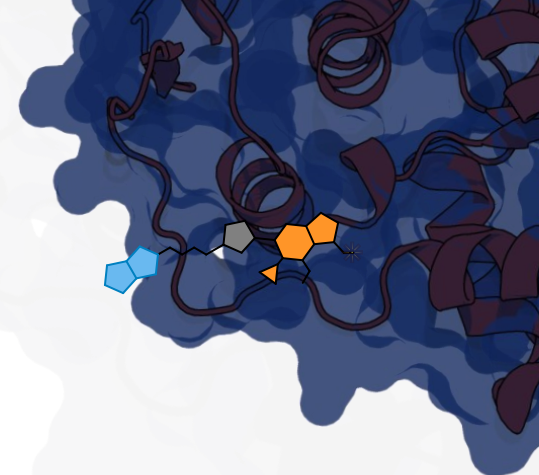
HIT & TELL

CHEMICAL PROBES TO MAP **PROTEIN** TARGETS
THROUGH AFFINITY-BASED PROTEOME PROFILING

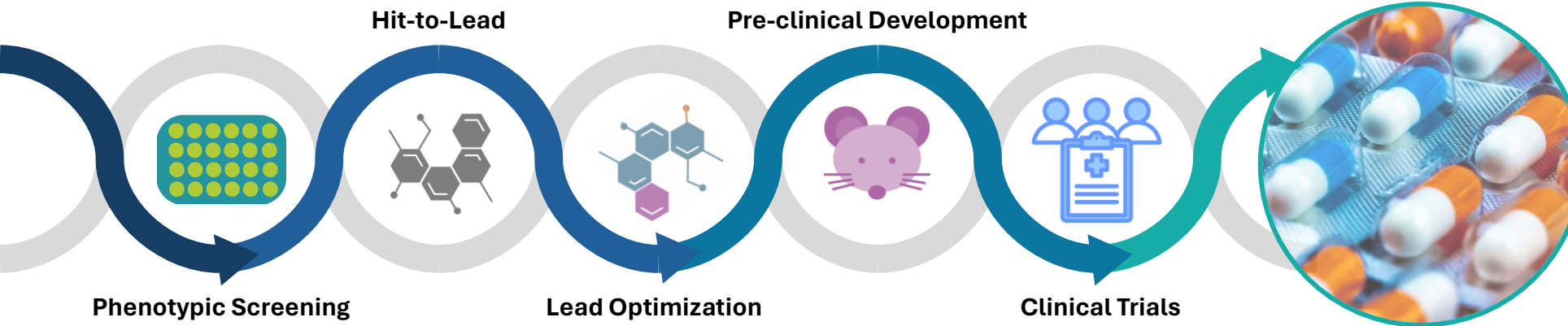
Lunch & Learn Webinar
Massimiliano Runfola
Wednesday 16 July 2025

OVERVIEW

- 1 Introduction to Protein Mapping**
Why understanding drug-proteome interactions is critical in modern drug discovery.
- 2 Affinity-Based Protein Profiling (ABPP)**
Principles and advantages for identifying protein targets in complex systems.
- 3 Designing Chemical Probes for ABPP**
Key considerations to ensure selectivity and reactivity.
- 4 ABPP Workflow: Tips and Tricks**
Practical insights to improve experimental design and data quality.
- 5 ABPP Applications**
Real-world examples demonstrating impact on target identification and drug development.



WHY IS IT IMPORTANT TO MAP PROTEIN TARGETS?



- **Phenotypic-first drug discovery** is reshaping early pipelines
- Target deconvolution allows **mechanistic understanding** that is **critical** for optimization
- **Defining the interactome reveals off-target risks**
- **Key to translational success**

A clear understanding of target engagement and off-target profiles supports confident progression into preclinical and clinical development

CHEMOPROTEOMIC APPROACHES FOR TARGET-ID

Key Principle

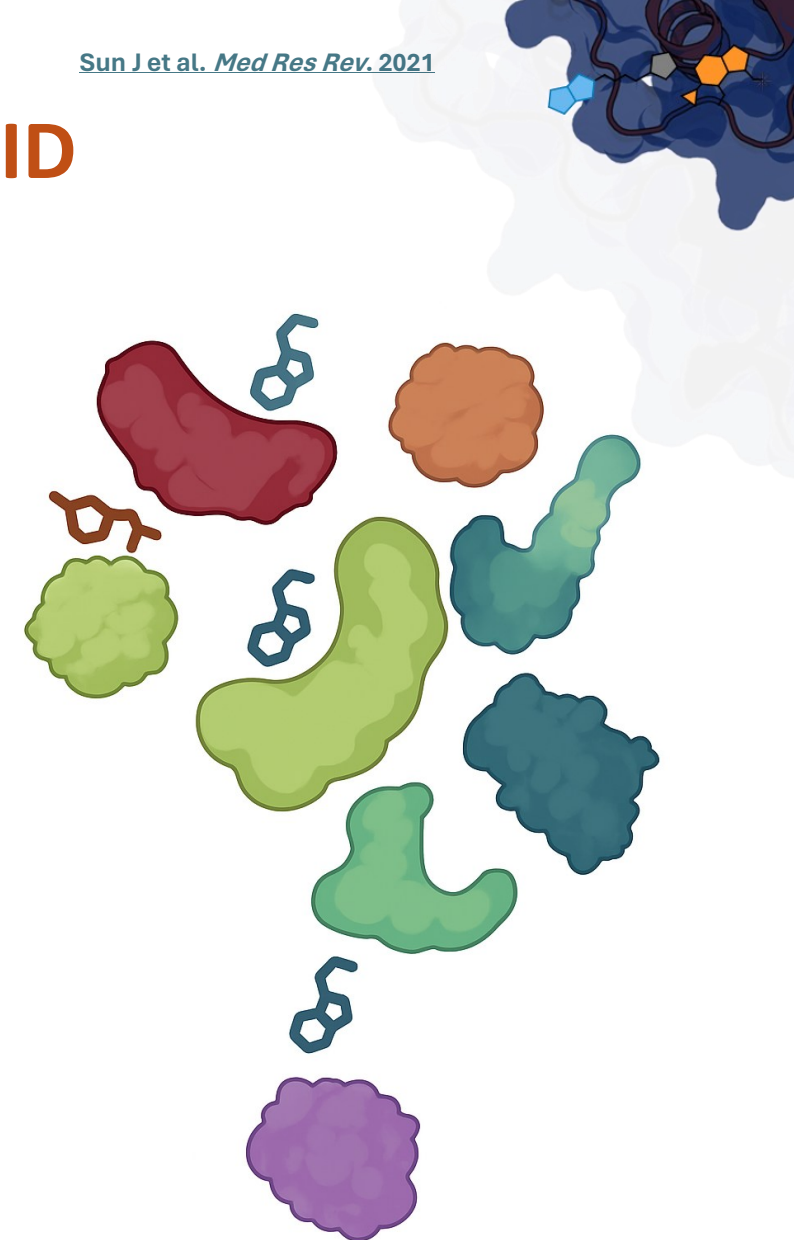
- Uses chemical probes or ligands to capture interacting proteins.
- Enables unbiased discovery of protein targets without overexpression or genetic manipulation.
- Allows study of target engagement in physiologically relevant contexts (e.g., in cells or tissues).

Advantages

- Captures **native protein conformations** and interactions.
- Can reveal **off-target effects**, providing important safety data.
- Suitable for **whole-proteome scale** studies.

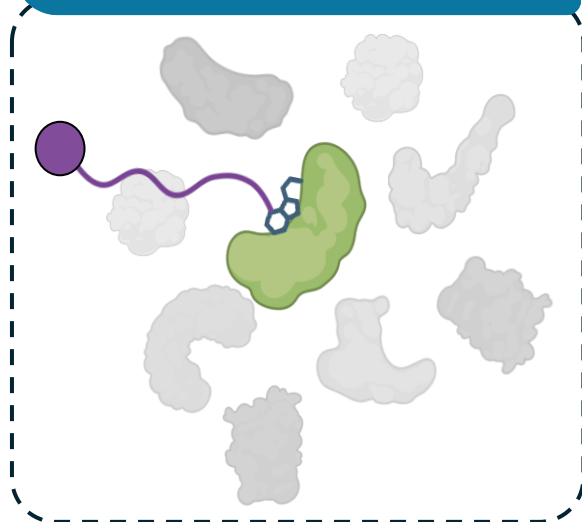
Main Approaches

- **Affinity-based**: direct fishing of targets using probes.
- **Stability-based**: detect changes in protein stability upon ligand binding.
- **Proximity-based**: capture proteins near the ligand.



CHEMOPROTEOMIC APPROACHES FOR TARGET-ID

Affinity-Based

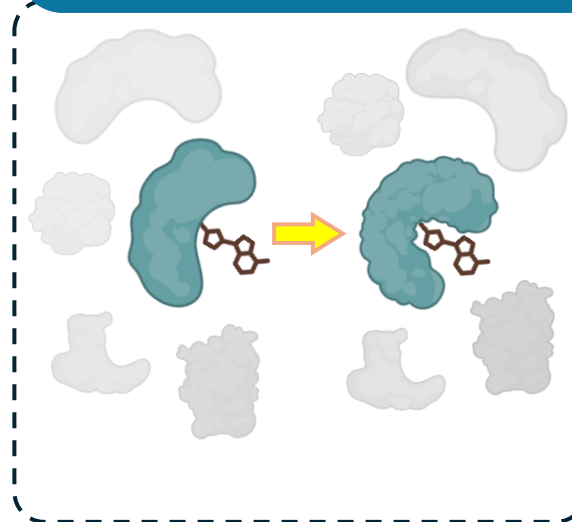


Modified probes to *fish* target proteins

e.g. ABPP

Requires synthesis of fine-tuned probes

Stability-Based



Detects biophysical changes upon ligand binding

e.g. MS-CETSA, DARTS, TPP

Label-free, but requires complex data analysis and can be very expensive

Proximity-Based



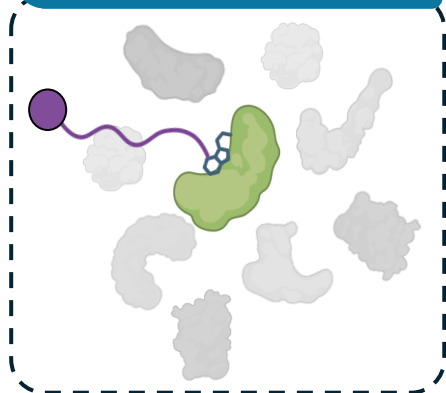
Enables labelling of proximal proteins using click-bait system

e.g. μ Mapping, REX

Labels all proximal proteins, not limited to binders

AFFINITY-BASED PROTEIN PROFILING (ABPP)

Affinity-Based



A chemoproteomic approach that uses **affinity probes** to capture proteins based on **specific binding interactions**.

Probes mimic small molecules with known activity to identify interacting proteins.

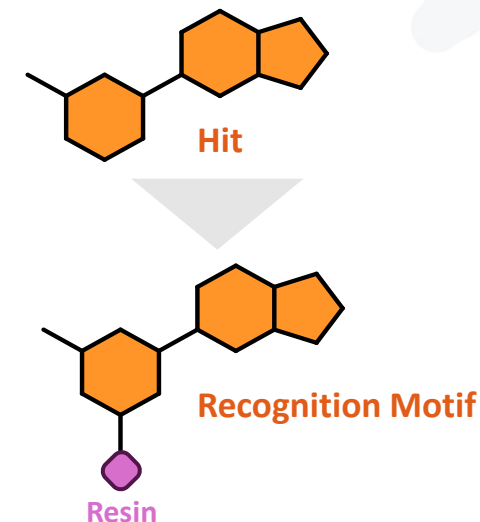
Requires binding small molecule to a resin for enriching protein targets

Applications

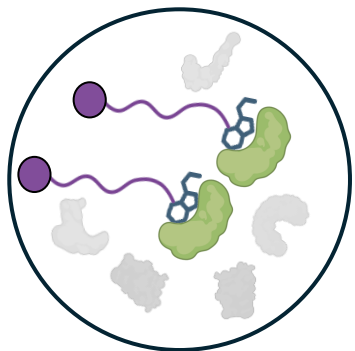
- Identifying drug targets and off-target proteins.
- Mapping protein-ligand interactomes.
- Studying metabolites signalling events
- Validating hits from phenotypic screens.

Advantages

- Direct physical binding detection.
- Works in **native** or near-native **proteomes**.
- Supports drug development and selectivity profiling.



EVOLUTION OF CHEMICAL PROBES FOR ABPP

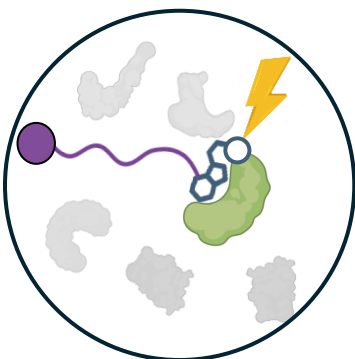


Early phase

small-molecules immobilized to resins

Used resin-bound small molecules to capture active enzymes.

- Enabled initial identification of targets but **limited** by poor accessibility and flexibility.

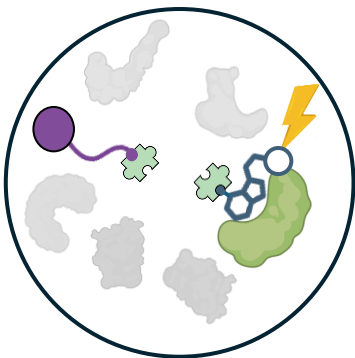


Photoactivable Labelling

small-molecules immobilized to resins & cross-linked to interacting proteins

Added photoreactive groups that upon UV light activation induce crosslinking of probes to interacting proteins, capturing transient or weak interactions.

- **Expanded probe applicability** to a wider range of proteins.



Bioorthogonal Chemistry

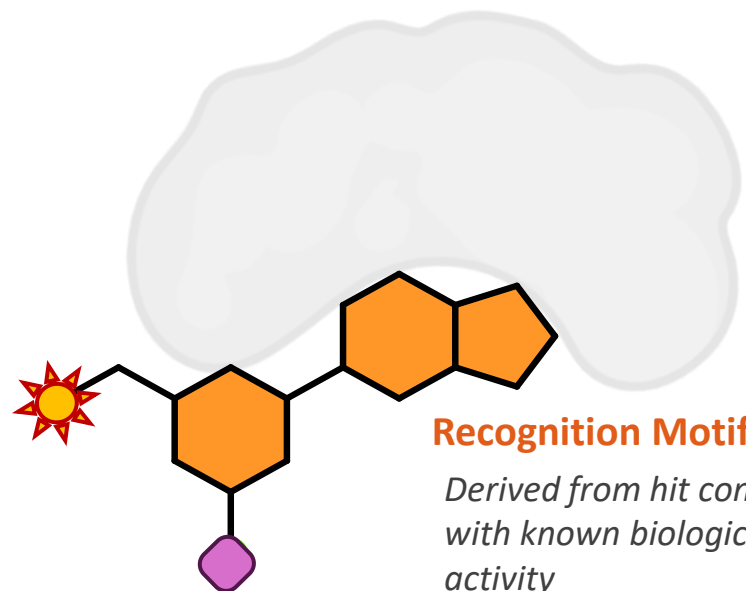
small-molecules cross-linked to interacting proteins, then clicked to resins

Designed **minimal probes without large detection tags**. After covalent labelling, tags (fluorophores, biotin) are attached via bioorthogonal click reactions. Reduced steric hindrance. Improved cell permeability. Lower background and higher versatility.

ANATOMY OF A MODERN PAL CHEMICAL PROBE

Reactive Group for Crosslinking

Allows to covalently bind the chemical probe to the target protein

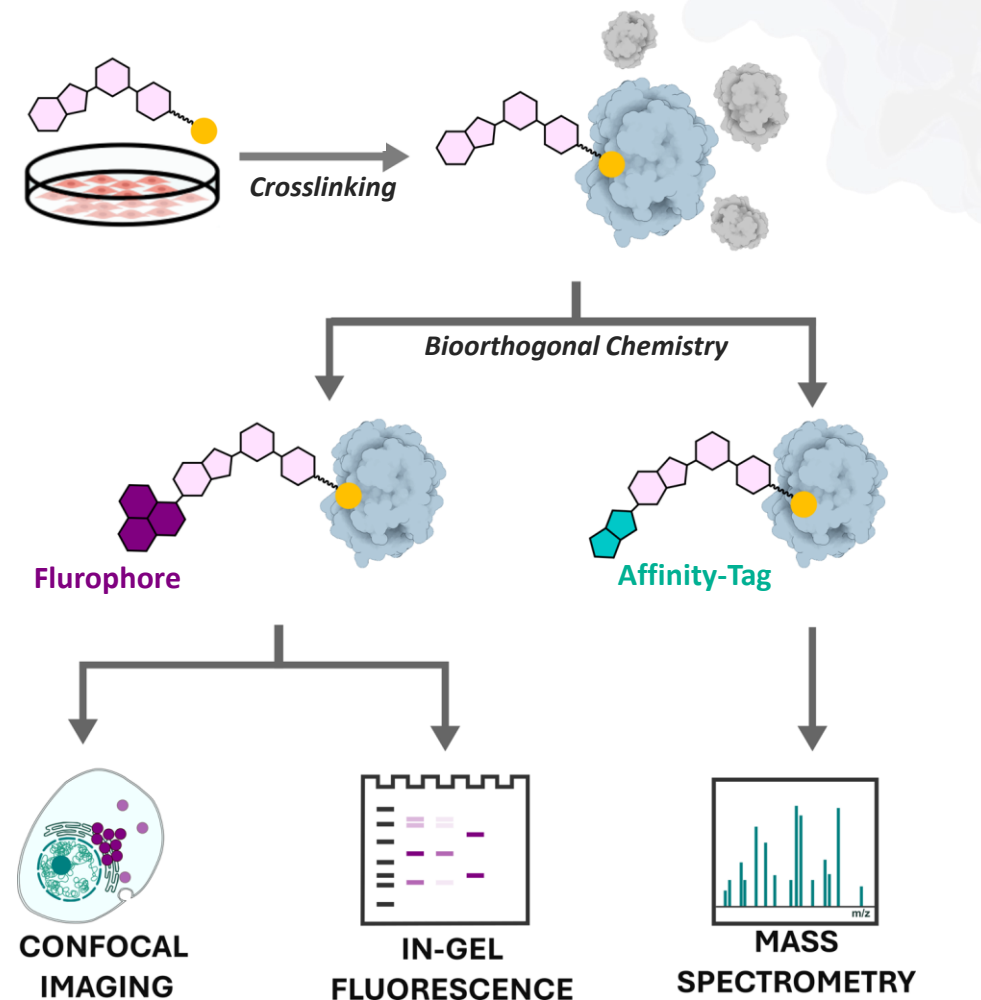


Recognition Motif

Derived from hit compounds with known biological activity

Clickable Group for Reporter

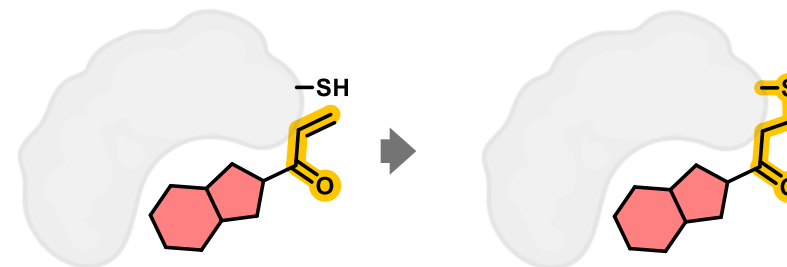
Probe-tagged proteins can be modified via click chemistry with a **fluorescent** reporter for visualization or an **affinity-tag** for enrichment



CROSSLINKING STRATEGIES

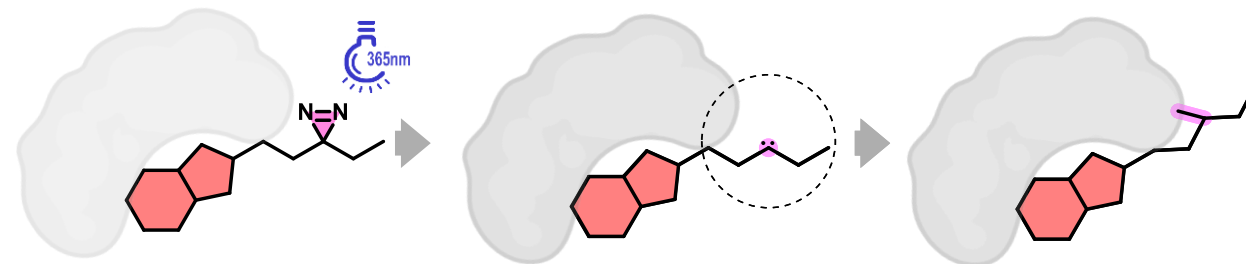
COVALENT WARHEADS (*Activity-based Protein Profiling*)

- Enables covalent labelling of chemical probe to interacting proteins directly on the binding site, exploiting nucleophiles in close proximity for covalent binding
- **Precise labelling of binding sites and no off-target proteins**
- **Easily applicable to covalent inhibitors**



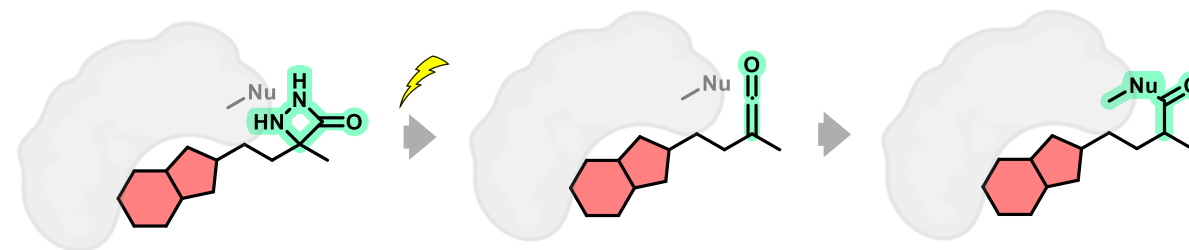
PHOTOAFFINITY LABELLING

- Enables covalent labelling of chemical probe to interacting proteins upon UV irradiation.
- **Allows temporal control of crosslinking**

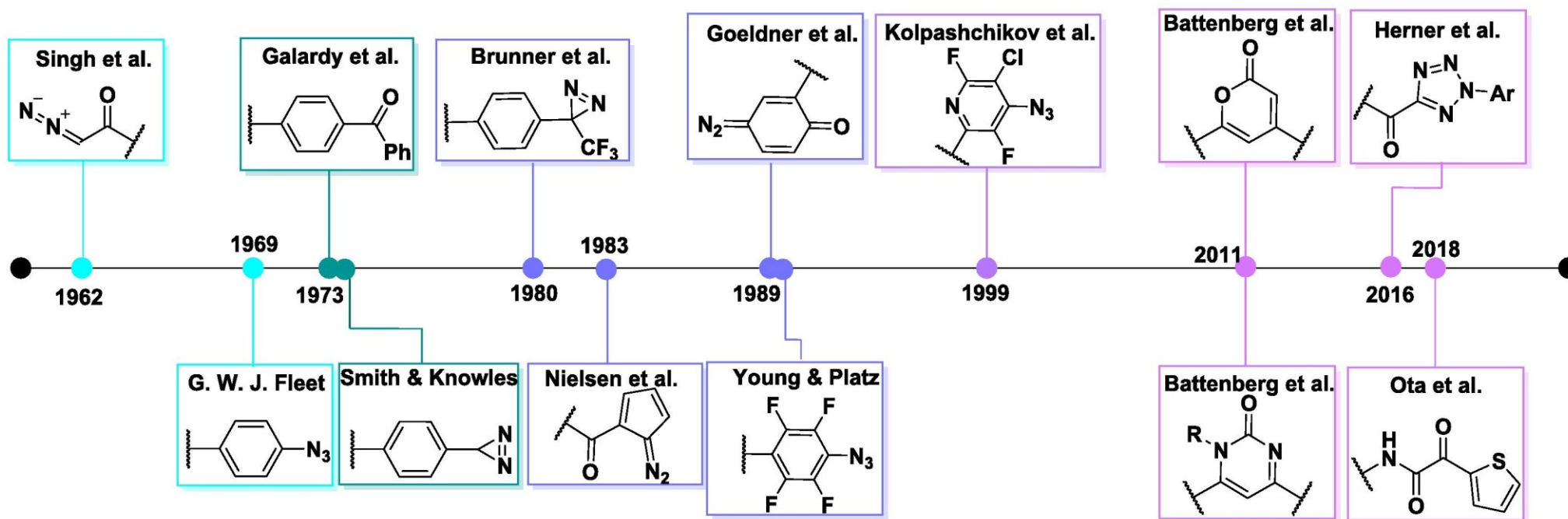
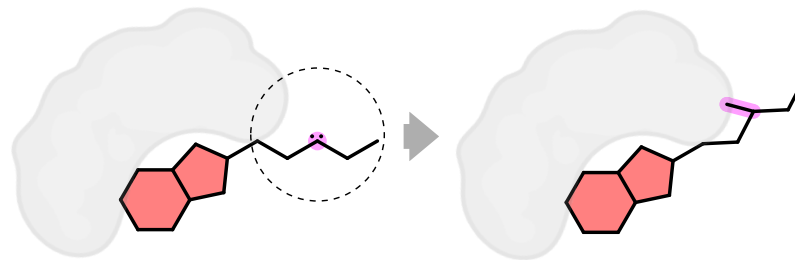


ELECTROAFFINITY LABELLING

- Innovative strategy that allows temporal control of the crosslinking applying a constant current of (+)C/(-)Ni for 1 min to oxidize a diazetidinone to ketene after N₂ extrusion
- **Highly tuneable**
- **Well-tolerated by cells**

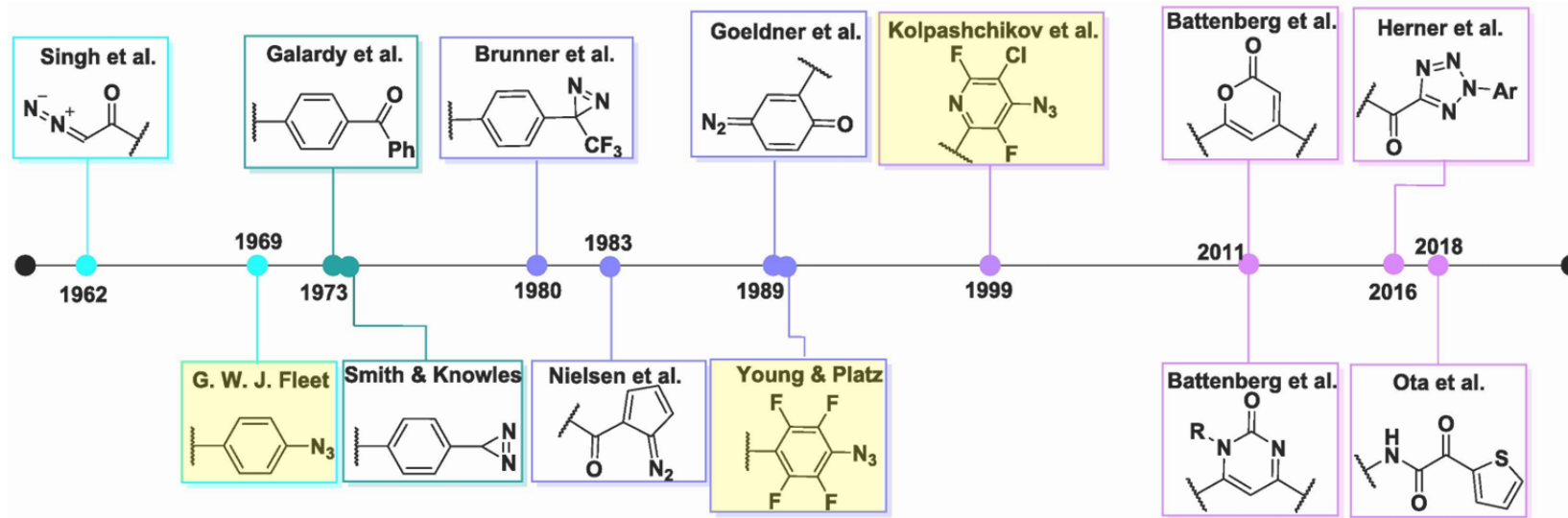
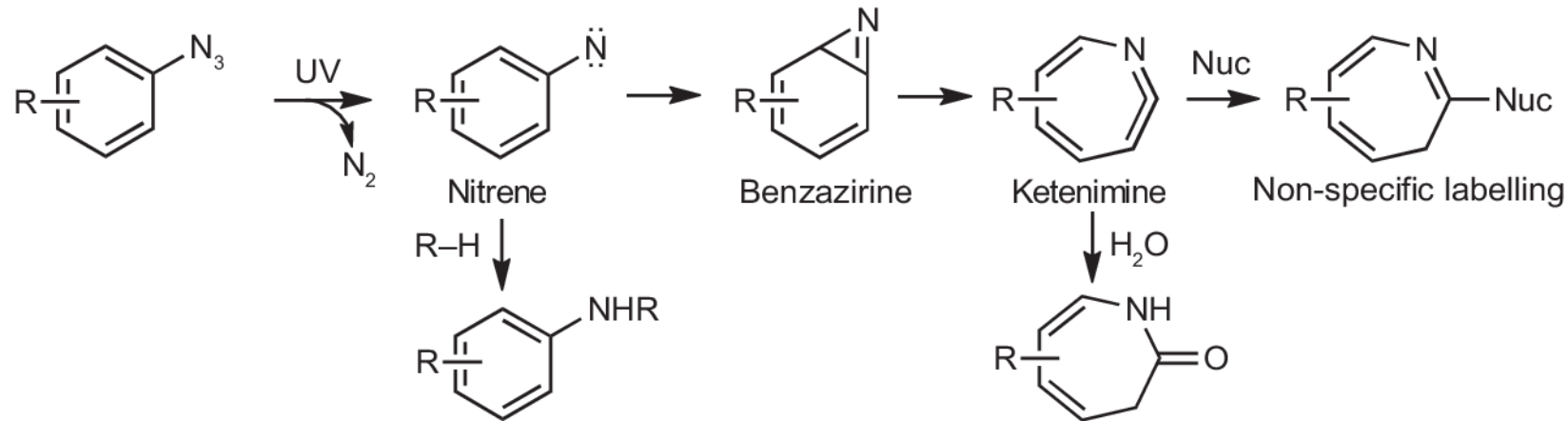


PHOTOACTIVABLE LABELLING



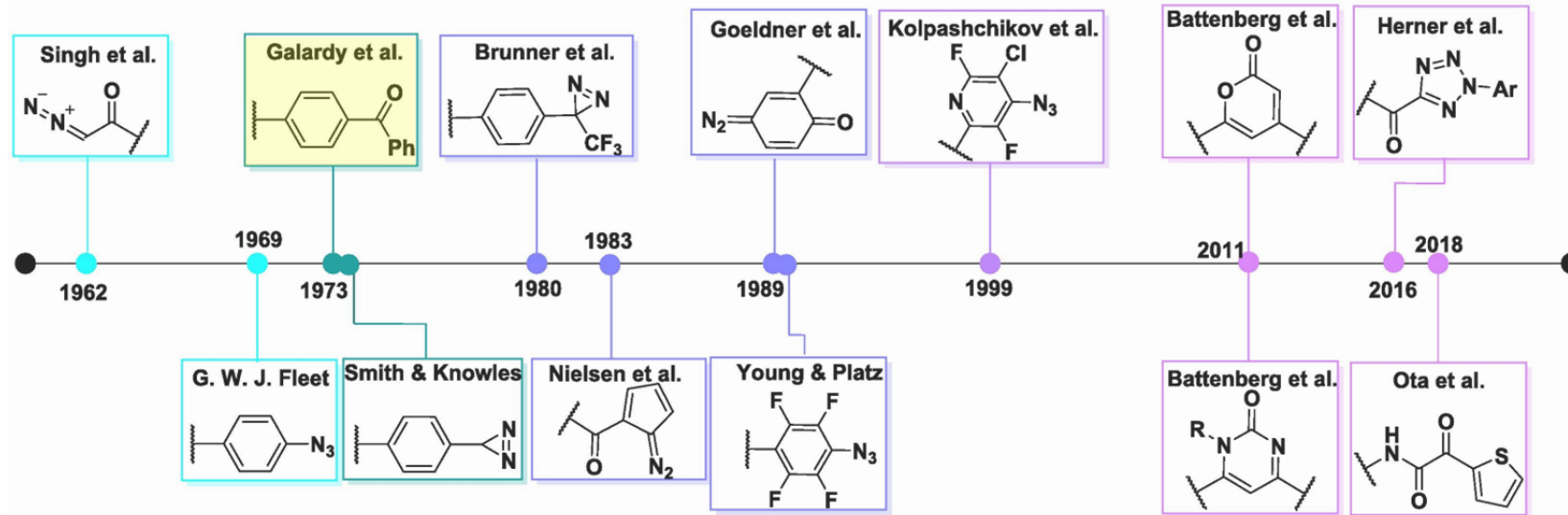
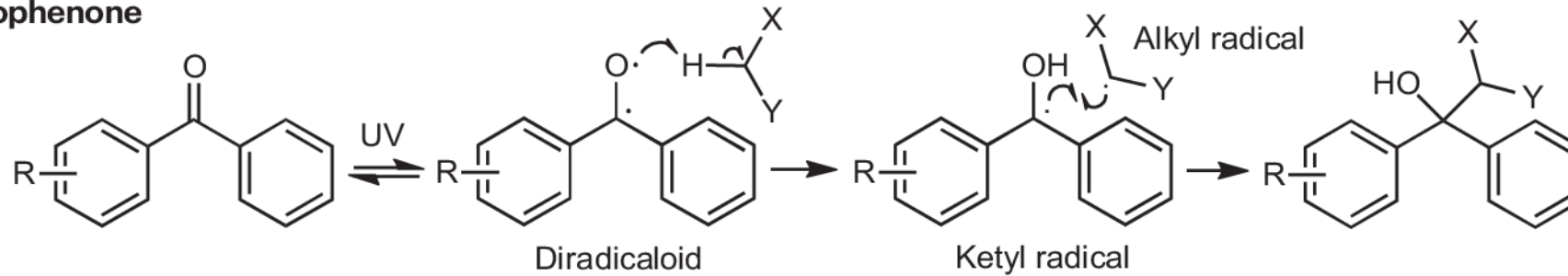
PHOTOACTIVABLE LABELLING

Aryl azide



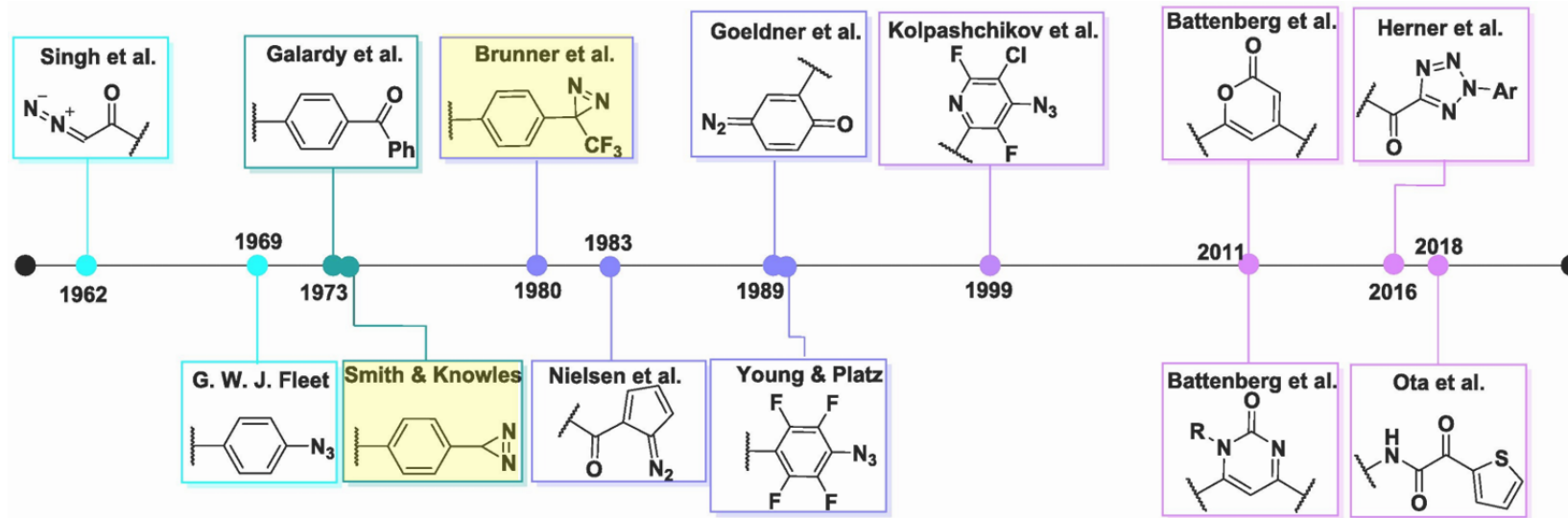
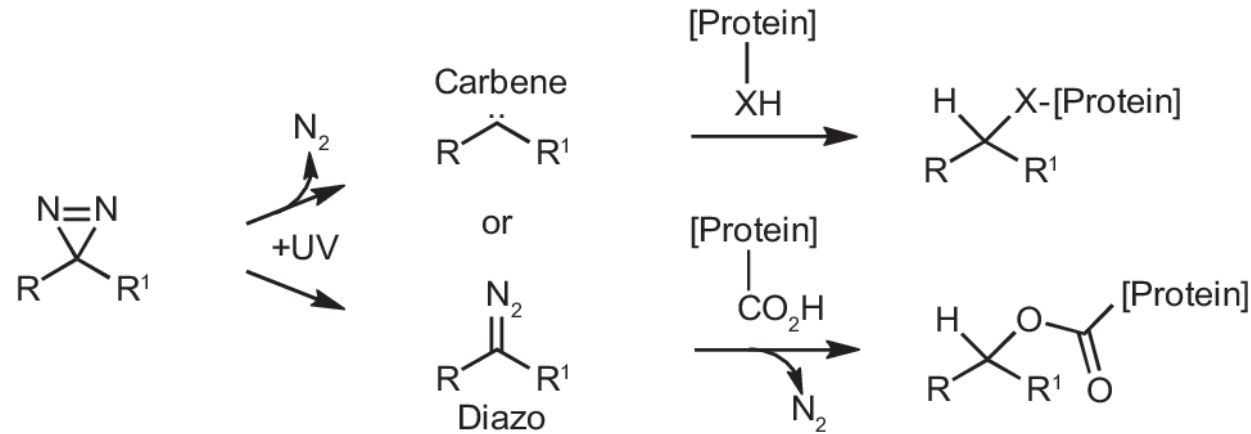
PHOTOACTIVABLE LABELLING

Benzophenone



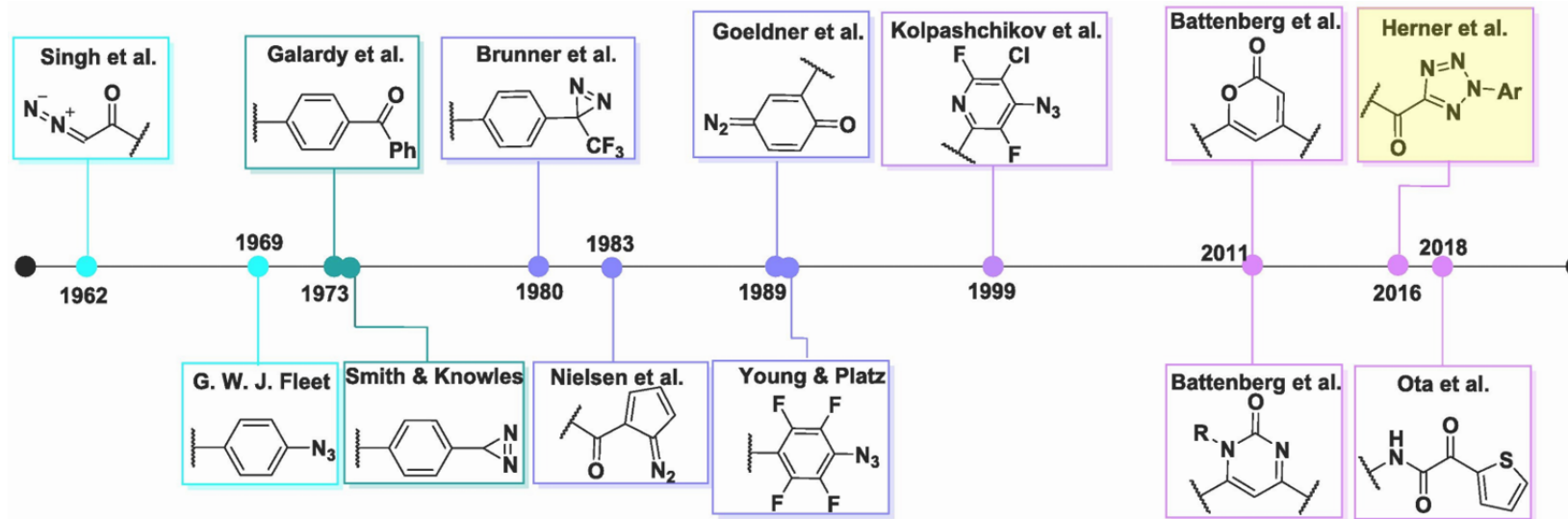
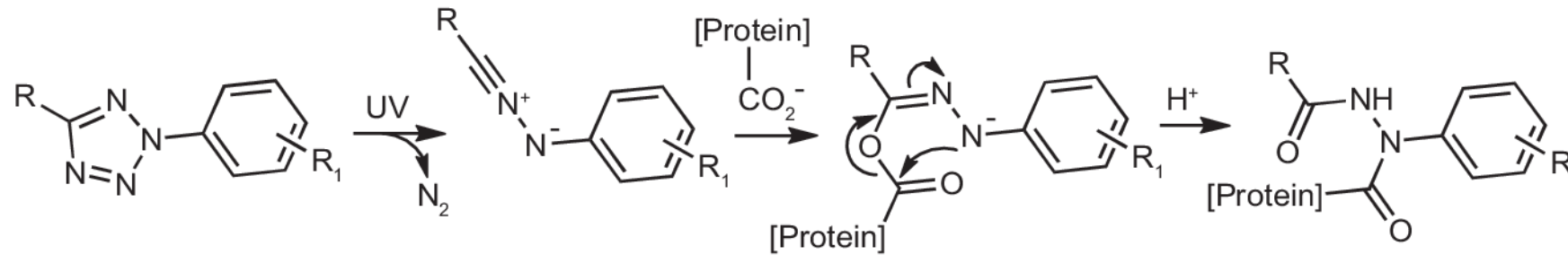
PHOTOACTIVABLE LABELLING

Diazirine

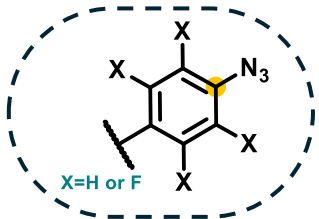


PHOTOACTIVABLE LABELLING

Aryl tetrazole



PHOTOACTIVABLE LABELLING



Aryl Azide

260-300 nm

Simple synthetic incorporation

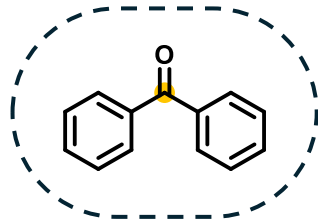
Small size

Nucleophile (nitrene)
 Cys (benzazarene)
 Lys (dihydroazepine)

High-energy photoactivation

Lower intrinsic reactivity

Side reactions with thiols



Benzophenone

350-365 nm

Simple synthetic conjugation

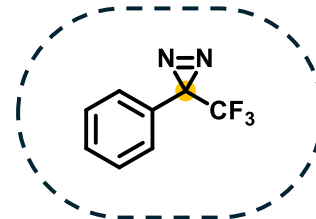
Reversible H₂O side reaction

Met

Bulky and high lipophilicity

Photosensitization may lead to DNA damage

Biased towards Met



Aryl trifluoromethyl Diazirine

~350 nm

Highly promiscuous reactive carbene

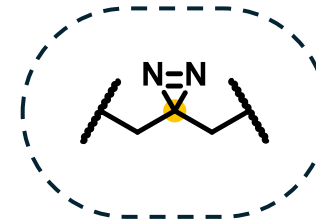
Reduced protein damage

None (carbene)
 Glu/Asp (diazo)

Bulky and lipophilic

Carbene quenched by water or O₂

Non-specific reactions



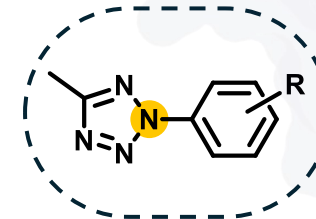
Aliphatic Diazirine

355-365 nm

Broadly reactive, minimal residue bias except with diazo intermediate

None (carbene)
 Glu/Asp (diazo)

Prone to diazo formation, rearrangements and non-productive reactions



Aryl Tetrazole

302 nm

Simple incorporation on carboxylic acid moieties

Labelling efficiency similar to diazirine

Glu/Asp

Highly selective for carboxylate side chains

Bulky and lipophilic

Higher-energy photoactivation

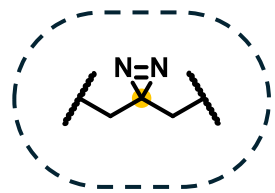
λ

ADVANTAGES

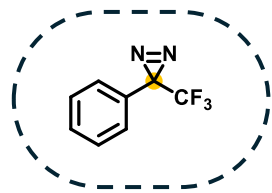
PREFERRED

DISADVANTAGES

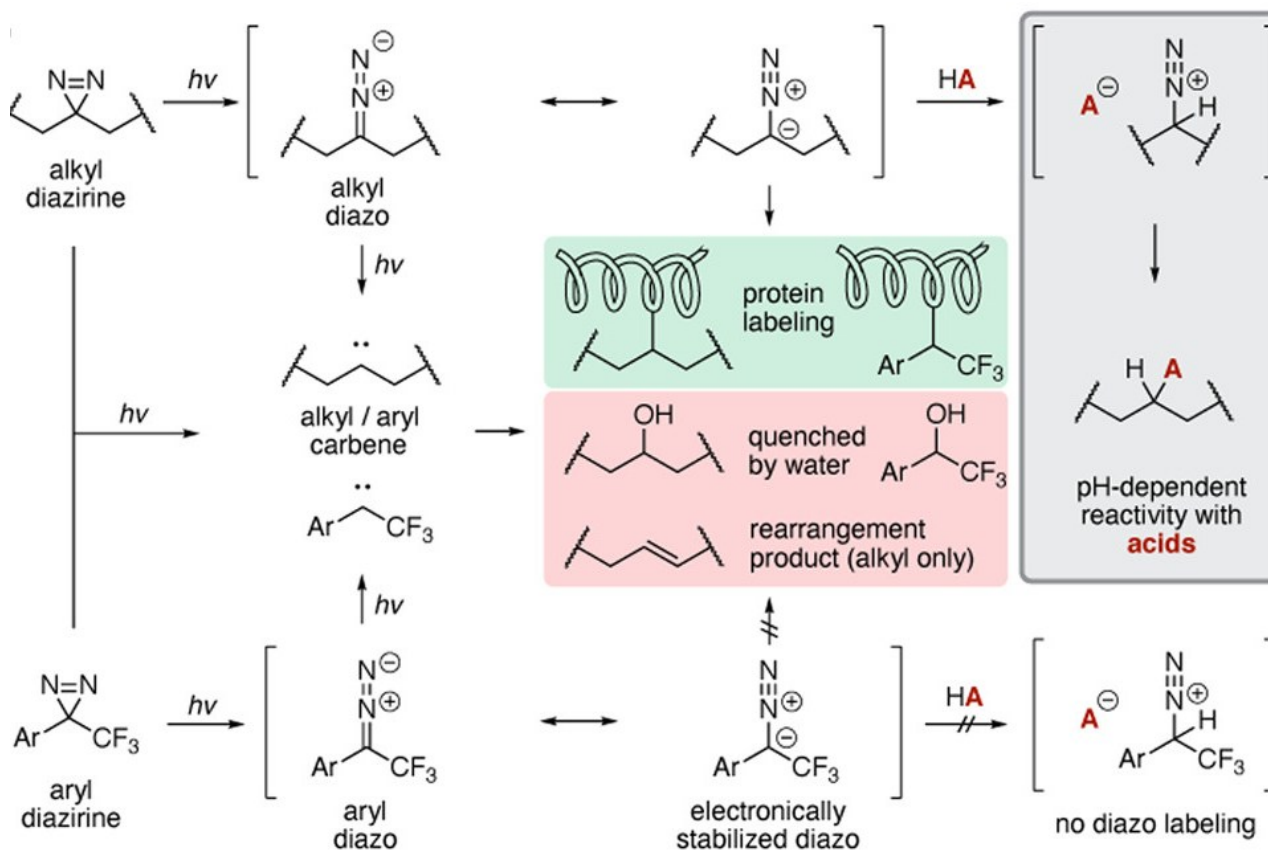
CROSSLINKING STRATEGIES: DIAZIRINE



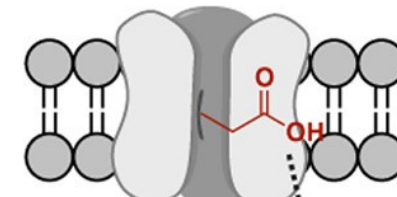
Aliphatic Diazirine



Aryl trifluoromethyl Diazirine

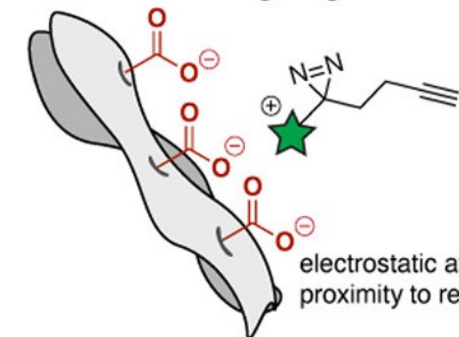


Membrane proteins



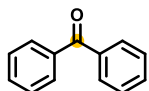
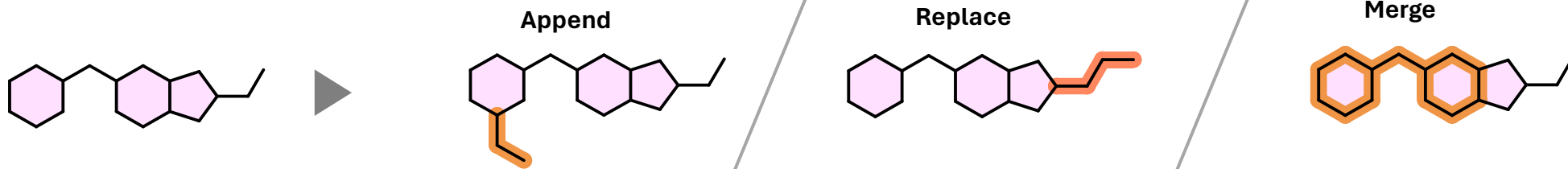
elevated pKa,
highly reactive protonated acids

Proteins with large negative electrostatic surfaces

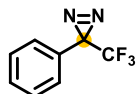


electrostatic attraction increases
proximity to reactive amino acids

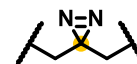
EXAMPLES OF PAL PROBES



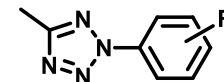
Benzophenone



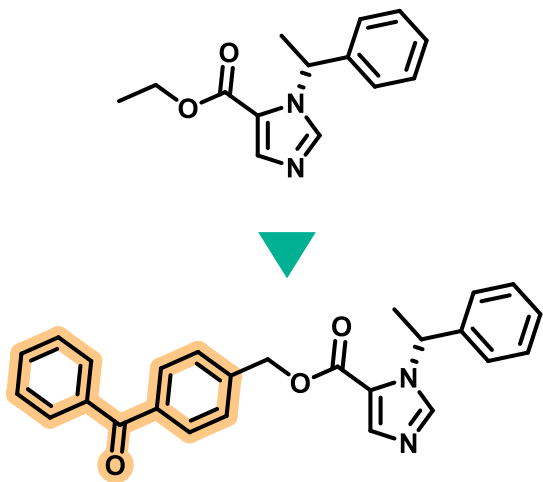
Aryl Diazirines



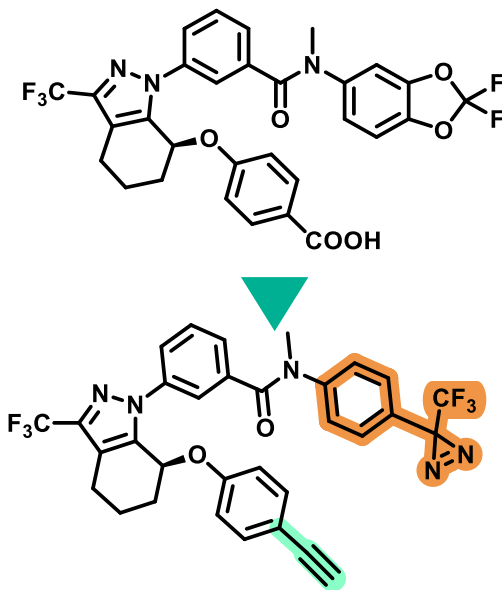
Aliphatic Diazirines



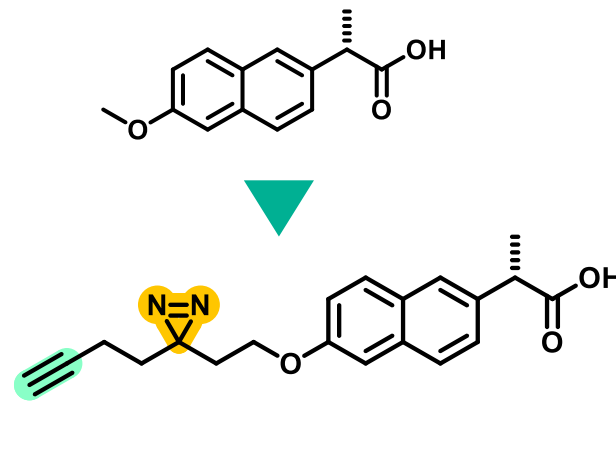
Aryl Tetrazoles



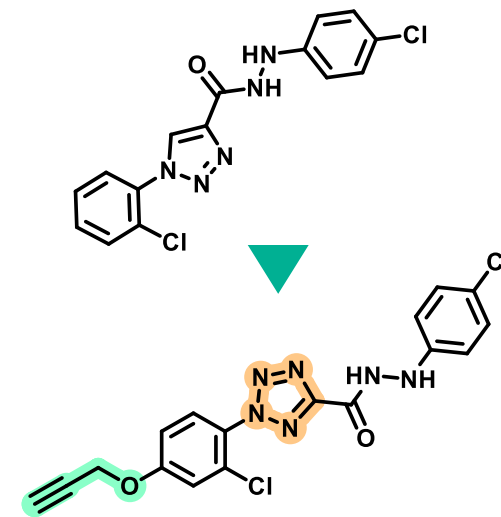
Husain SS et al, J Med Chem, 2006



Romeo E et al, J Med Chem, 2025



Gao J, J Am Chem Soc, 2018



Liu J, J Agr Food Chem, 2025

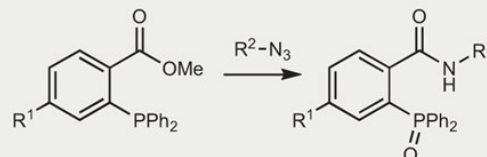
BIOORTOGHONAL CHEMISTRY

high-yielding chemical reactions that proceed rapidly and selectively in biological environments without side reactions towards endogenous functional groups

Reactants	Product
Carbonyl condensation $\text{H}_2\text{N}-\text{X} + \text{R}(\text{H})-\text{C}(=\text{O}) \rightarrow \text{R}(\text{H})-\text{C}(=\text{N}-\text{X})$ $\text{X} = \text{NH}, \text{O}$	
Modified Staudinger ligation 	
Traceless Staudinger ligation 	
Inverse-electron demand Diels-Alder cycloadditions Strained/activated 	
Copper-catalyzed azide-alkyne cycloaddition (CuAAC) 	
Strain-promoted azide-alkyne cycloaddition (SPAAC) 	

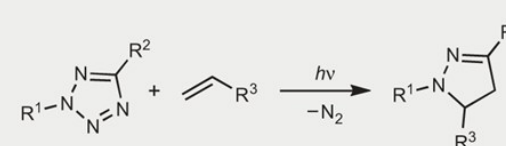
Staudinger ligation

In vivo formation of amide bonds



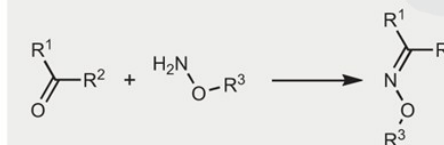
Photoinducible bioorthogonal chemistry

Spatio-temporal control with light



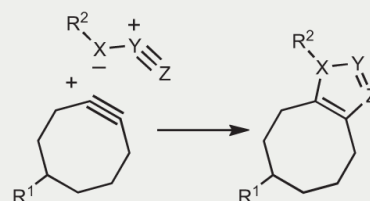
Oxime and hydrazone ligations

Enabled by ketone and aldehyde tags



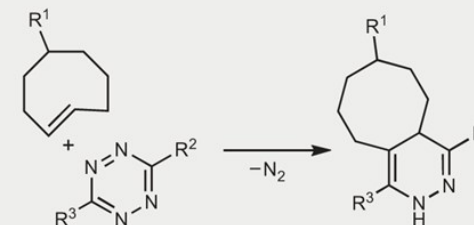
Strain-promoted [3 + 2] cycloaddition

Copper-free coupling with 1,3-dipoles



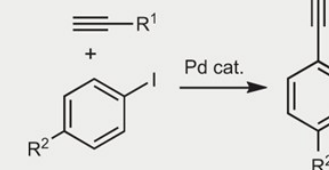
Tetrazine ligation

Exceptionally rapid kinetics



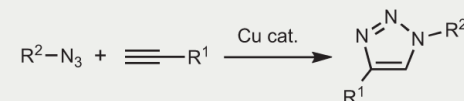
Palladium and ruthenium-catalysed couplings

Unique tags for biomolecule modification



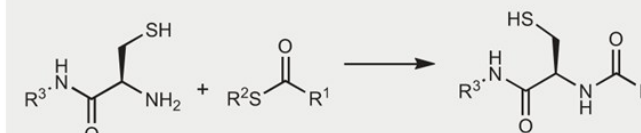
Copper-catalysed azide-alkyne cycloaddition

Minimal size, readily accessible, broadly useful



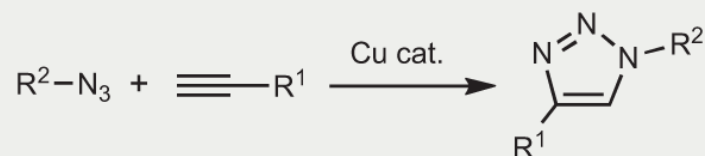
Native chemical ligation

Protein synthesis from synthetic or expressed fragments



BIOORTOGHONAL CHEMISTRY

CuAAC



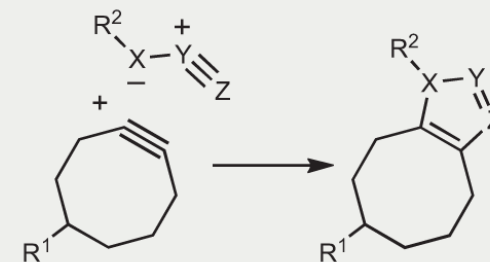
Copper-catalysed azide-alkyne cycloaddition

- **Small, minimal perturbation of molecular function.**
- **Largely unreactive in biological systems**
- Modular, aqueous-compatible, by-products free-, user-friendly
- Done after lysis to avoid live-cell copper toxicity
- Allows enrichment & detection of active proteins

Limitations

- *Some proteins can still be damaged by copper in lysates*
- *Thiols may inactivate copper catalyst, reducing efficiency*
- *Requires careful control of reaction conditions and copper ligands*

SPAAC



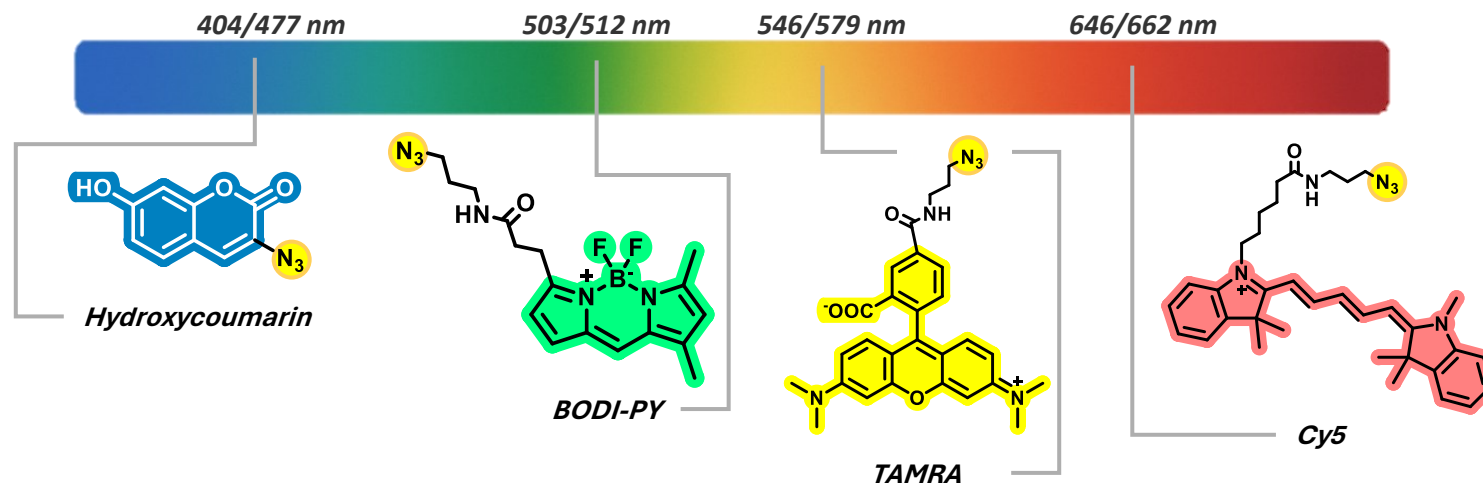
Strain-promoted [3+2] cycloaddition

- **Copper-free alternative using strained alkynes**
- **No copper catalyst needed**
- Avoids copper-related damage to sensitive proteins
- Compatible with lysates and live-cell applications
- Simplifies workflow, no need for copper ligands or quenching steps

Limitations

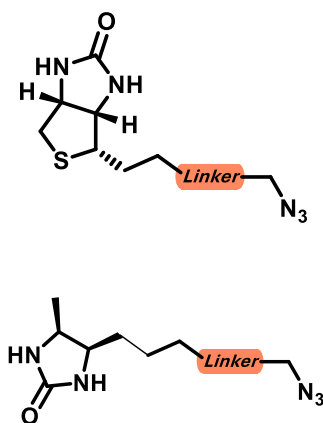
- *Strained alkynes are bulkier, may affect probe design or reactivity*
- *Slower than CuAAC in some contexts*

COMMON REPORTERS



Fluorophores

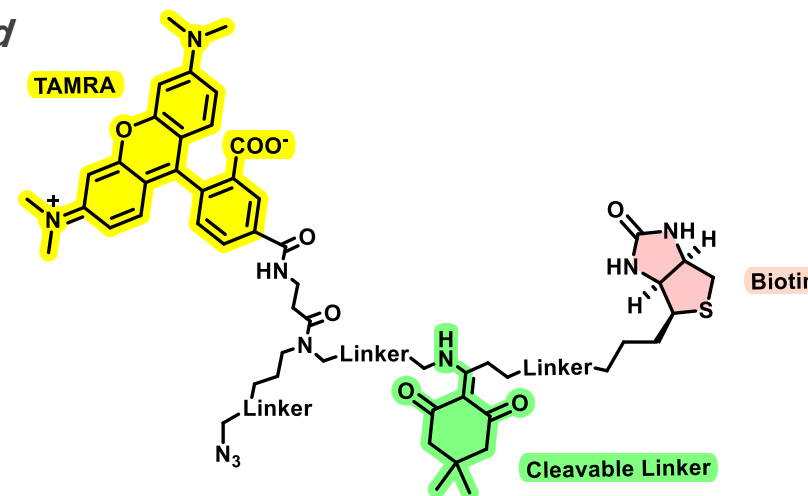
- Wide range of commercially available fluorophores
- Selection depends on application (e.g., excitation/emission wavelengths, brightness, photostability)
- Commonly used in ABPP for visualization, target validation, and quantification



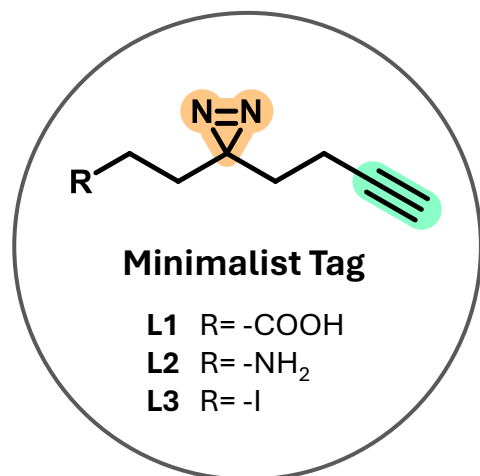
Affinity-tags

- Biotin-azide available with various linkers (application-dependent)
- Preferred choice for enrichment experiments
- Biotin-streptavidin interaction provides very strong binding
- Cleavable linkers can be incorporated as needed

Combined

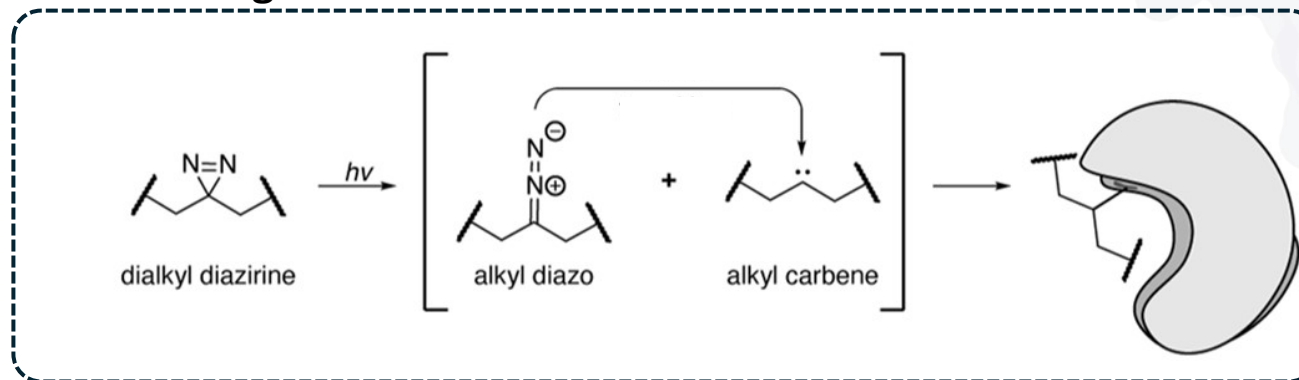


ADVANCES IN PROBE DESIGN

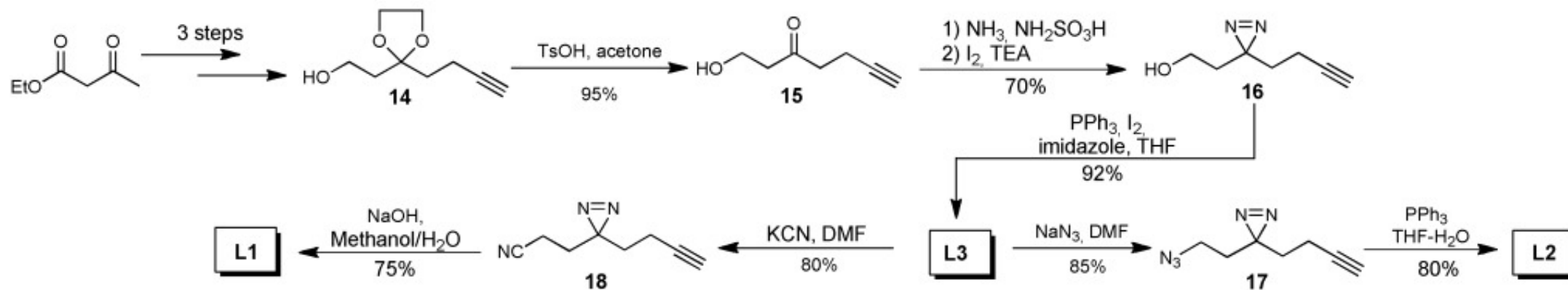


- *Minimalist tag*
- *>8 steps synthesis*
<30% overall yield
- *Could be easily inserted as last step*
- *Stable*
- *60-200£ per 50mg*
- *pH-dependent*

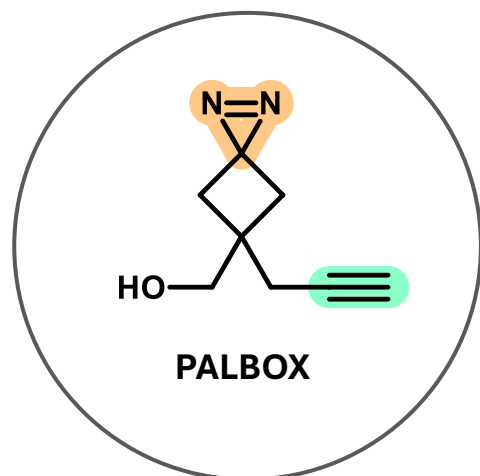
Crosslinking



Synthesis

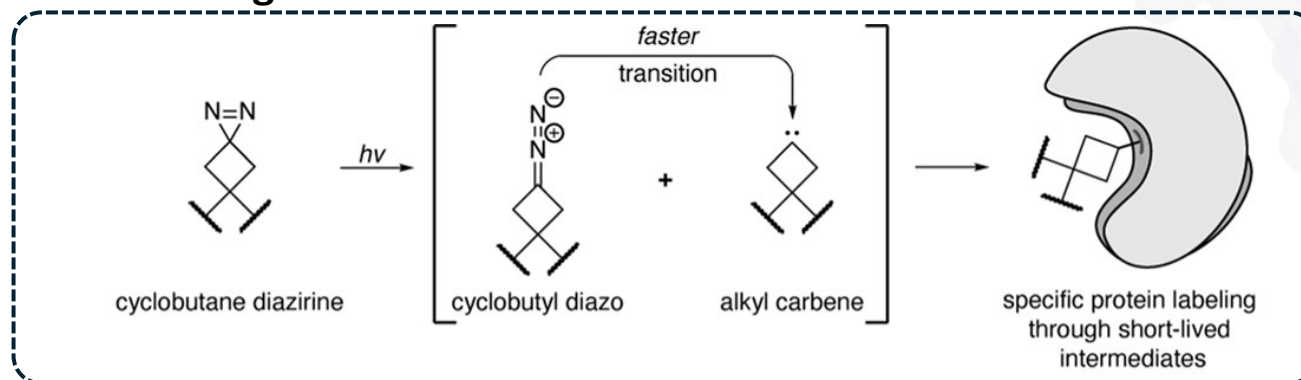


ADVANCES IN PROBE DESIGN

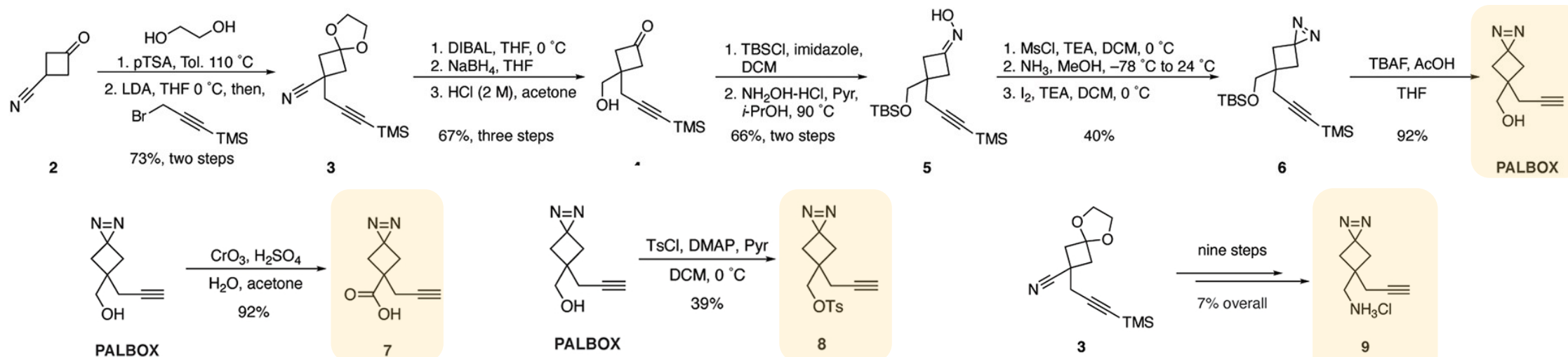


- *Minimalist tag*
- *11 steps synthesis*
12% overall yield
- *Could be easily inserted as last step*
- *Stable*
- *pH-independent*
- *Lengthy and expensive synthesis*

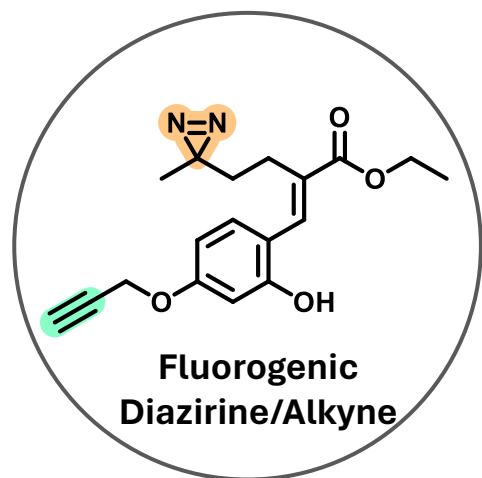
Crosslinking



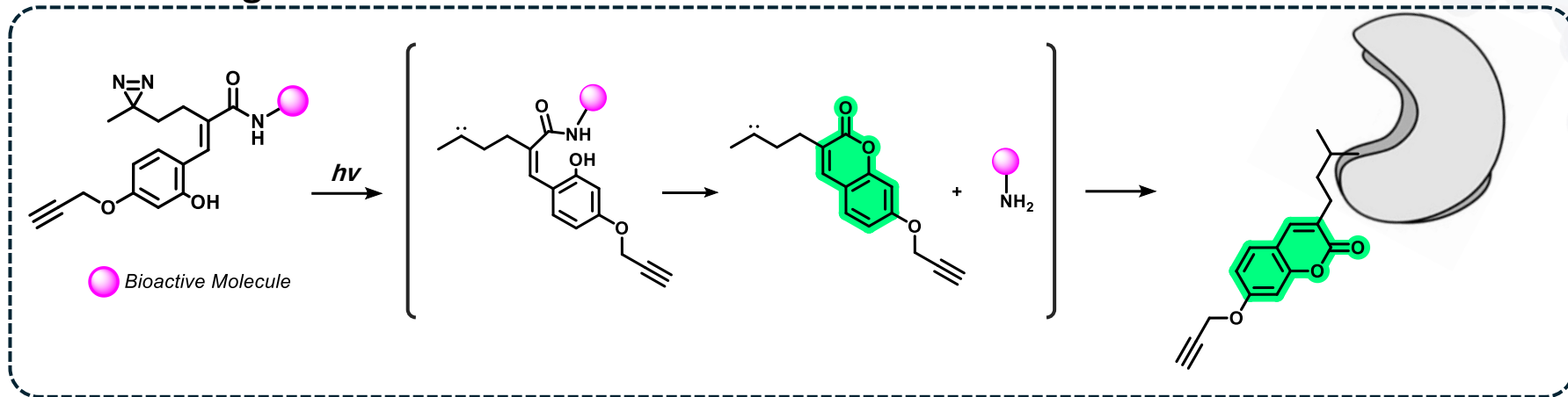
Synthesis



ADVANCES IN PROBE DESIGN

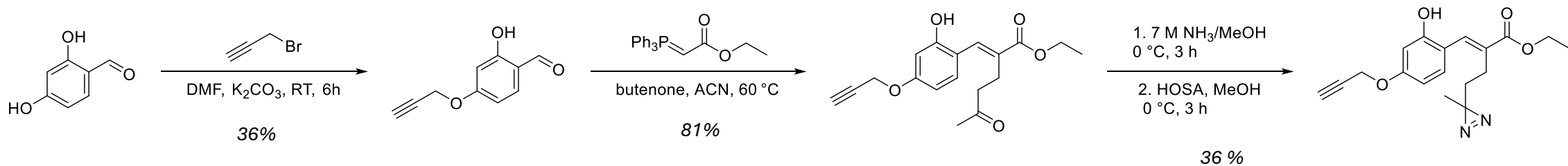


Crosslinking



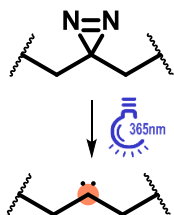
- *Interesting approach*
- *4 step synthesis*
11% overall yield
- *Could be inserted as last step*
- *Quite bulky could affect probe-target interactions*

Synthesis



VALIDATING THE PAL PROBE

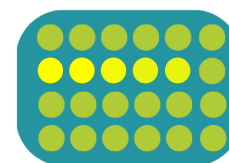
Photoactivable Crosslinking



NMR or LC/MS

1. Dissolve probe in MeOH or MeOD
2. Irradiate with UV light for several minutes
3. Check formation of Methoxy-adduct via NMR or LC/MS

Biological Activity



Phenotypic assay

Validate probe biological behaviour on same phenotypic assay(s) used for identifying hit compound (dose-response)

Labelling Efficiency



In-gel Fluorescence

1. Treat cells from phenotypic assay with probe and UV-irradiate.
2. Lyse cells, click reaction with fluorophore
3. Run SDS-PAGE
4. Verify protein are labelled in right conditions

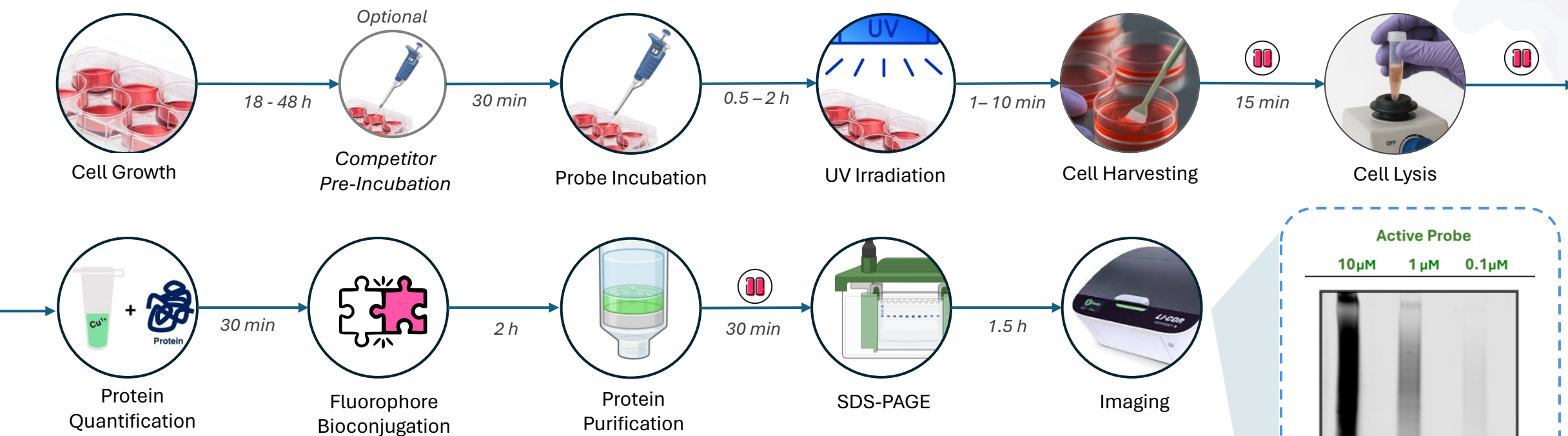
Labelling Specificity



Competition Assay

1. Pre-incubate cells with structurally similar competitor (e.g. hit compound)
2. Treat cells with probe, irradiate, lyse and click reaction.
3. Run SDS-PAGE
4. Verify dose-dependent disappearing of labelled proteins

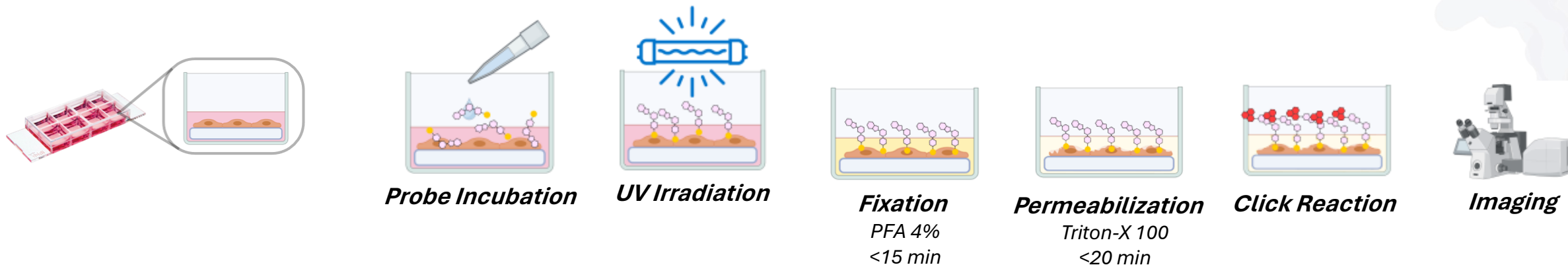
ABPP EXPERIMENTAL WORKFLOW



- Critical part as it provides information on labelling specificity of the probe, establishes the protocol for proteomics, provides information on potential protein hits MW.

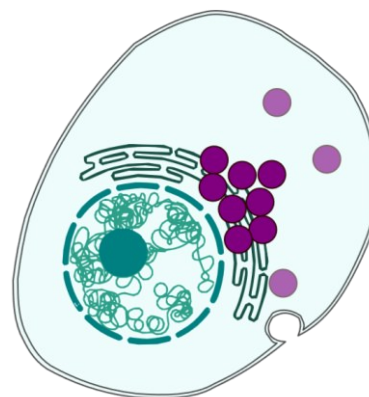
- Protocol needs to be adapted to **cell line**, **probe mechanism**, and available **instruments**.

CELLULAR VALIDATION: IMAGING

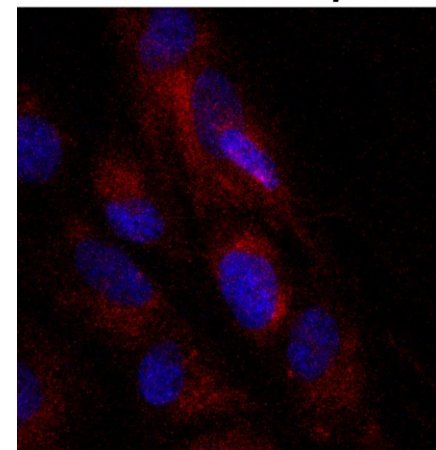


- Useful to validate probe entering cells, if UV-irradiation time is damaging cells, etc
- Allows understanding of probe localization
- Can be used for competition assays and for target engagement studies using appropriate antibody

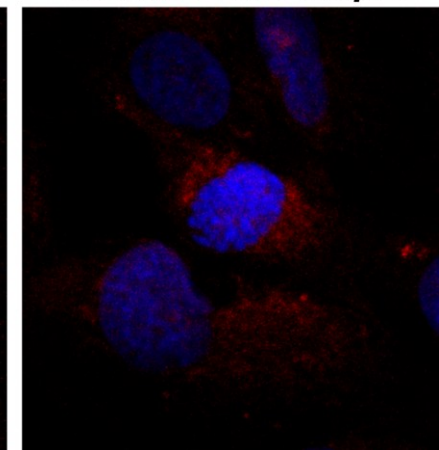
Example



Active Probe 1 μ M



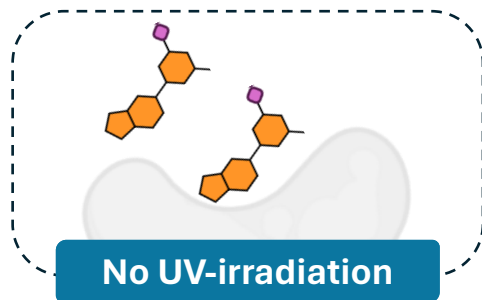
Active Probe 0.1 μ M



CONTROLS FOR ABPP

Using adequate controls is critical for success of ABPP.

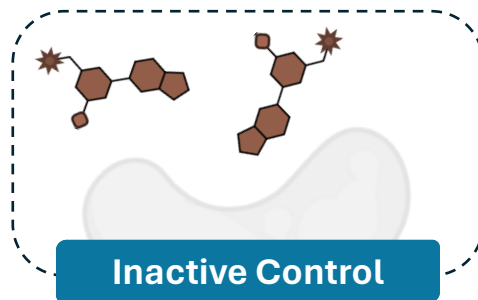
The right choice of controls depends on specifics of project aims and hit biological profile



Skip irradiation step on active probe treated cells.

Non-specific photo-crosslinking and background labelling

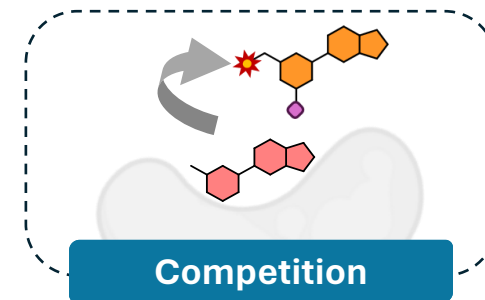
Helps confirm signal dependence on probe reactivity.



Use structurally similar but non-reactive analogues.

Validates specificity of probe-target interactions

Demonstrates that labelling is activity-dependent



Pre-incubate with excess of known inhibitors or parent

Assesses selectivity and target engagement

Demonstrate labelling at binding sites.

TRANSITION TO CHEMOPROTEOMICS

Probe biologically validated for specificity and sensitivity

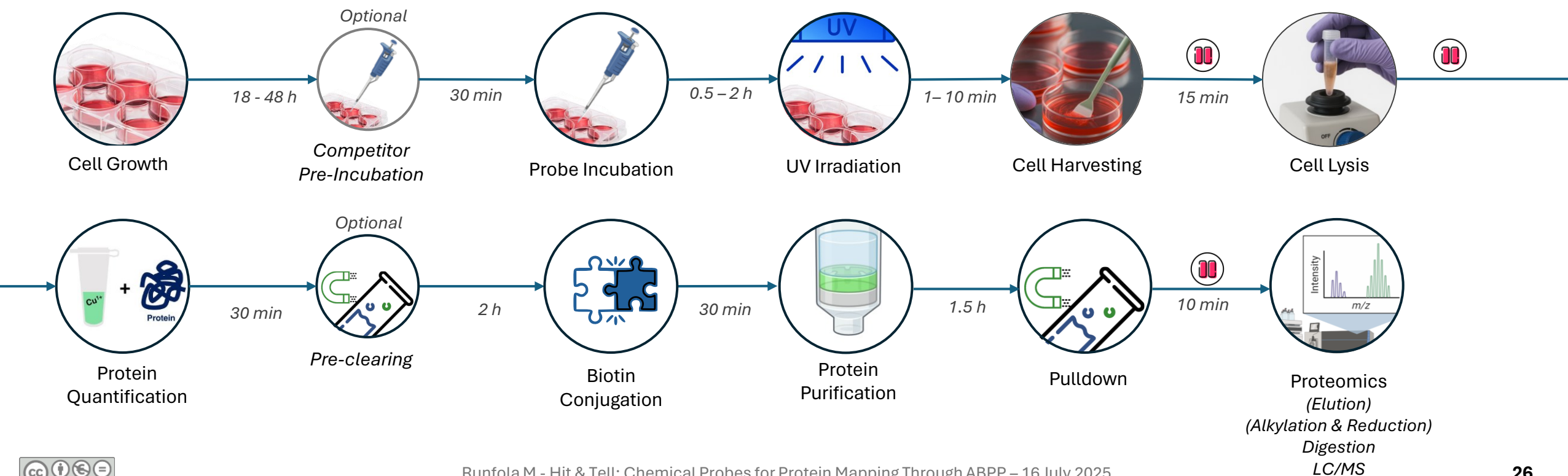
Adequate controls

In-gel Fluorescence provides information on size of protein

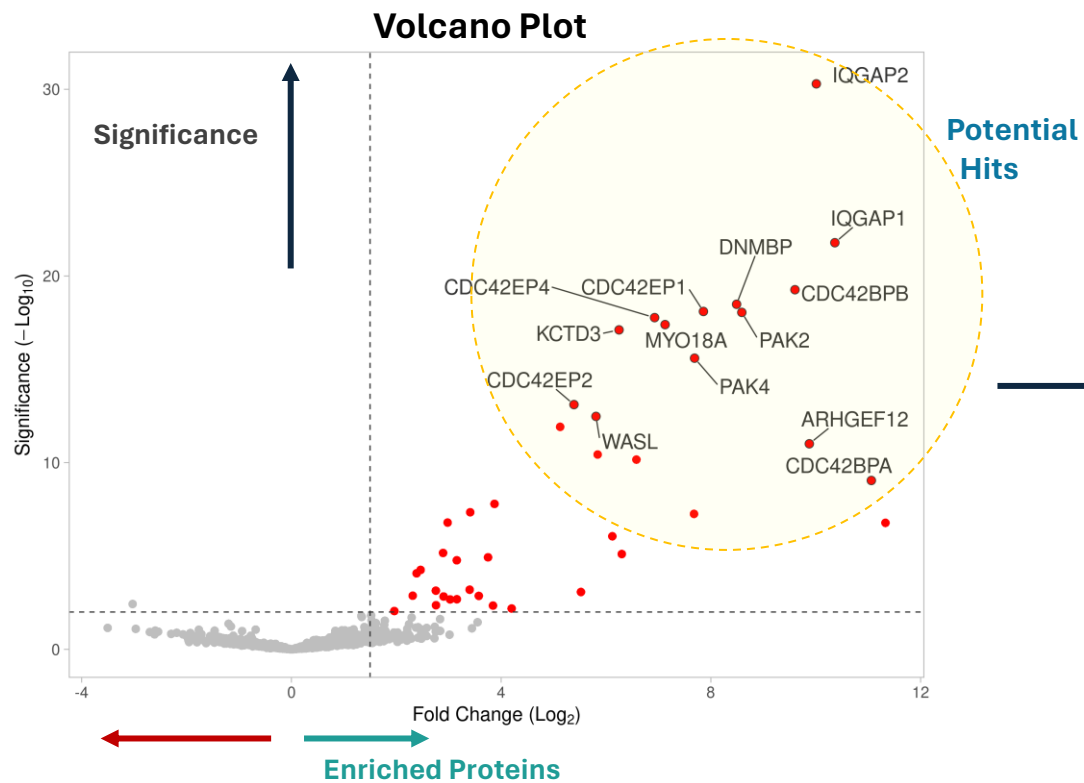
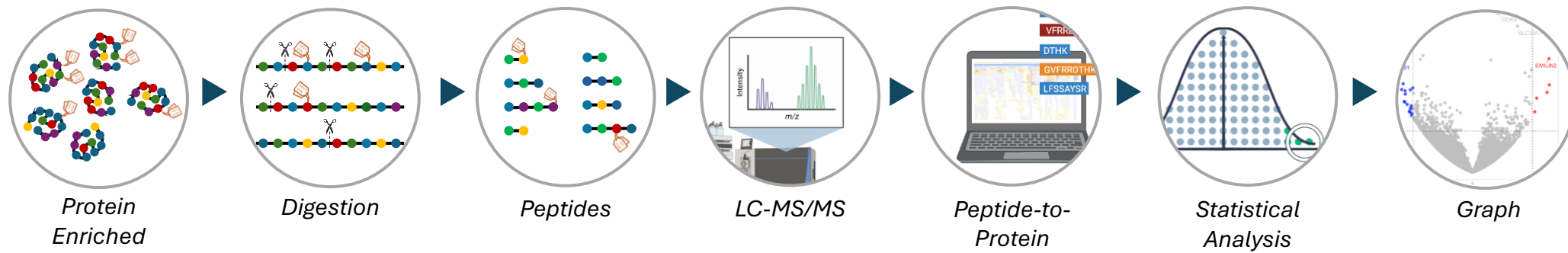
Confocal imaging provides information on cellular localization of probes

Ready for moving to MS-Proteomics

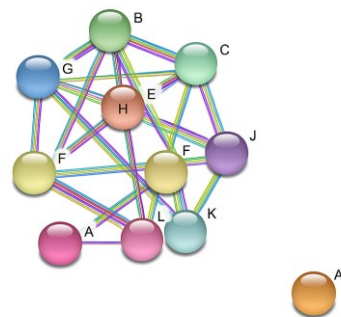
*Similar workflow, but **fluorescent reporter is substituted with biotin** to allow enrichment via streptavidin binding*



DATA ANALYSIS & BIOINFORMATICS



STRING



Protein Molecular Weight
Cellular Localization
Protein Network Analysis
Protein Enriched

Data collected

Select Protein Hits

Validate by Biology/Pharmacology

- *Western Blot*
- *CETSA*
- *Confocal Imaging (competition)*

ABPP APPLICATIONS: TARGET DECONVOLUTION

DMD



Severe and progressive **muscle wasting disease**



2500 people living with DMD in UK



1 in 3500 live male births



7000+ mutations on DMD gene



Wheelchair by the age of 10



Death by cardiac or respiratory arrest **early adulthood**

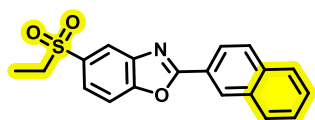
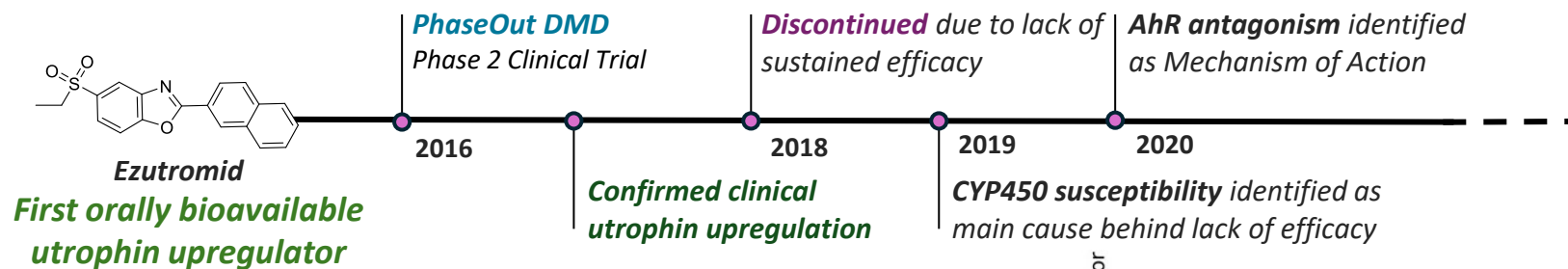
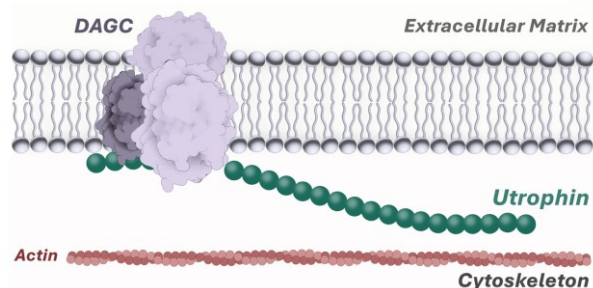


Gower's Maneuver

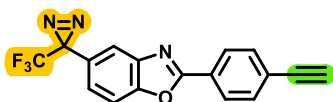


Learning and walking difficulties

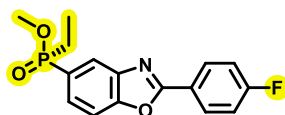
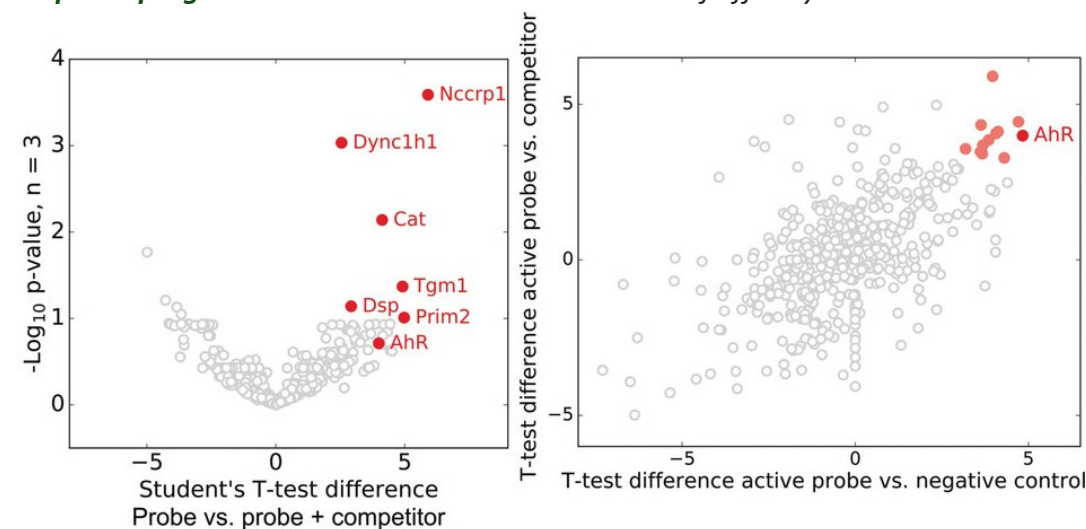
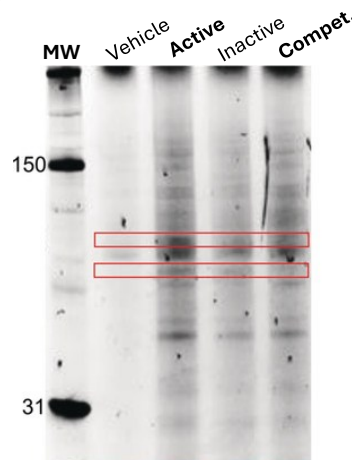
UTROPHIN UPREGULATION



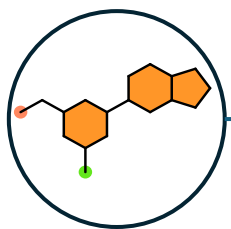
Ezutromid



Probe

2nd Gen

TROUBLESHOOTING & RECOMMENDATIONS



Probe Design & Synthesis

Clearly define SARs to determine where to insert the PAL group and alkyne.

Use multiple PAL groups for improved results.

Do not rely on a single probe; the more options, the better.

Choose straightforward synthetic strategies, and whenever possible, **opt for commercially available tags**.



Biological Validation

Approach validation **methodically**.

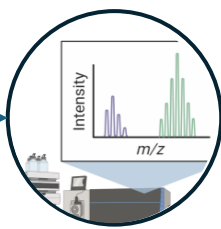
Optimize timing for treatment (< 2 h).

Test more than one cell line or model for robust validation.

Remember that **each model is unique**

Be flexible. Adapt the protocol to your needs

Remember: at this stage, **no result is "bad"**, all results are informative.



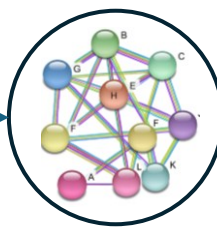
Chemoproteomics

Use multiple controls

When possible, keep a **high number of biological replicates** (ideally $n \geq 4$).

Validate protocols using in-gel fluorescence and WB until confident.

Test different conditions and probes for comprehensive insights.



Data Analysis & Hit selection

Reference academic literature, it is your best tool.

Use free-online resources for data analysis and graphing.

Discuss findings with colleagues from diverse backgrounds.



Target Validation

Utilize all collected data for planning validation experiments.

Prioritize simple and efficient approaches that yield clear answers.

Remember:

If something goes wrong, an error in the process is controllable and often due to human oversight.

Double-check your steps!

| CONCLUSIONS & TAKE-HOME MESSAGE



ABPP is a versatile and powerful platform for drug discovery (at all stages!)

Enables precise interrogation of small molecules behaviour in biological systems

Accelerates the identification and optimization of therapeutic candidates and disease-relevant targets

Broad potential applications

Target Deconvolution

Off-target profiling

Binding Sites Mapping

Screening systems

Target Validation

Functional assays (*e.g. cellular localization, target engagement*)

Embrace Biology

Understand the pharmacology behind testing small molecules is critical

THANKS FOR YOUR ATTENTION

