Introduction:

Tetrazines readily react with strained or vinyl alkenes in a highly selective and bioorthogonal way. All **ATTO tetrazines** are based on **6-methyl-3-aryl tetrazine (MeTet)** which provides high stability in aqueous media and still shows very high reaction rates. The ligation of **ATTO tetrazines** with e.g. trans-cyclooctenes (TCO) proceeds with rate constants of up to 1000 M-1 s -1 , about three orders of magnitude faster than typical azide alkyne cycloaddition (AAC) or strain promoted azide alkyne cycloaddition (SPAAC). This high reactivity is a prerequisite for any application performed under highly diluted conditions such as protein conjugations.

The TCO-tetrazine ligation can be considered as a strain promoted inverse electron demand DielsAlder cycloaddition (SPIEDAC), forming a dihydropyridazine derivative[1] (Figure 1).

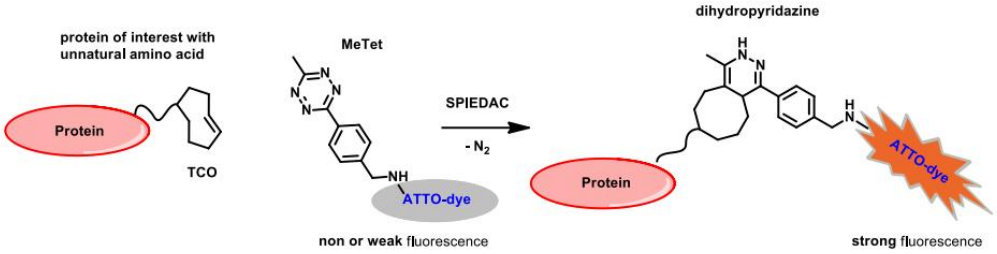

Figure 1: TCO-tetrazine ligation with ATTO-dye labeled tetrazine.

For many ATTO-dyes the fluorescence is heavily quenched by the tetrazine residue. After the conjugation reaction has taken place the initially strongly reduced emission is restored, making them fluorogenic probes[2]. This is reflected by turn on ratios of up to a factor 30 depending on the dye and reactant.

ATTO tetrazines are available for a variety of fluorophores. They are provided in units of 0.2 and 0.5 mg (Table 1).

Protocol for TCO-tetrazine ligation:

The following protocol describes the labeling procedure for **ATTO tetrazines** with TCO labeled biomolecules, e.g. proteins.

Required Materials

• **Component A:** Dissolve the **ATTO tetrazine** in the appropriate amount of DMSO to obtain a 0.5 - 1 mM solution. Aliquots of this solution may be stored at -20 ° C. Note: The shelf life of such solutions will be significantly reduced depending on the quality of the solvent used.

• **Component B:** The TCO-carrying protein should be dissolved in a buffer

like PBS, HBSS (Hank's balanced salt solution) or in case of transfected cells in an appropriate cell growth medium, e.g. DMEM (Dulbecco's modified Eagle medium).

Tetrazine conjugation

• Pipette the appropriate amount of **Component A** to **Component B** to achieve a final tetrazine concentration of 1 - 3 μM.
• Incubate for 10 - 30 min at 4 ° C, 25 ° C or 37 ° C depending on the application.
• After successful ligation, excess amount of **ATTO tetrazine** can be washed away using PBS, HBSS or DMEM (2-3 times). In the case of fluorogenic tetrazines, like ATTO 425 MeTet, ATTO 465 MeTet, ATTO 488 MeTet, ATTO 490LS MeTet, ATTO 532 MeTet and ATTO 655 MeTet, the washing step may be omitted.
• Cell fixation can be carried out with 4 % formaldehyde in PBS for 15 min followed by three wash cycles with HBSS.
• No fixation for live cell staining

Table 1: ATTO-dye labeled tetrazines:

Dye	MW	M+	λ abs	λ em	ε max
ATTO 425	585	586	439	485	45000
ATTO 465	593	479	453	506	75000
ATTO 488	887	773	500	520	90000
ATTO 490LS	879	857	495	658	40000
ATTO 532	943	829	532	552	115000
ATTO 540Q	842	742	543		105000
ATTO 550	891	777	554	567	120000
ATTO 565	794	694	564	590	120000
ATTO 590	874	774	593	622	120000
ATTO 594	1003	989	603	626	120000
ATTO 647N	930	830	646	664	150000
ATTO 655	825	711	663	680	125000
ATTO 680	823	709	681	698	125000

MW: molecular weight of the dye including counterions in g/mol; M^+ : molecular weight of dye cation (HPLC-MS acetonitrile/water 0.1 vol-% trifluoroacetic acid); λ abs: longest wavelength absorption maximum in nm; λ em: fluorescence maximum in nm; ε max: molar decadic extinction coefficient at the longest-wavelength absorption maximum in $M^{-1}cm^{-1}$.