**In situ labeling and TMT preparation**

In situ treatment

1. Cells grown to 80-95% confluency in 10cm dishes in growth media
2. Media aspirated and cells washed w/cold DPBS (2x)
3. Incubated w/ 2 mL of serum-free media containing probe for 30 min at 37°C

In situ crosslinking prep

1. Media aspirated and then cells irradiated for 10 min under 365-nm UV light in Stratagene UV Stratalinker 1800 at 4°C (no lid).
2. Cells collected by scraping, and transferred to eppendorf tubes and centrifuged
at 3000 rpm for 3 min, remove supernatant.
3. Cold DPBS (1 mL) added to each eppie, vortex to resuspend pellet.
4. Repeat steps 2 & 3 (\*cell pellets can be stored at -80°C at this stage or as lysates)
5. Add cold DPBS (~400 µL) to pellet
6. Cell pellets were lysed by sonication and protein concentrations determined by using the BCA protein assay on a microplate reader. Protein concentrations adjusted to ~1 mg/mL (500 µL). Note: If pellet is to be fractionated, after sonication, lysate fractionated by centrifugation (100,000g, 45min) to yield soluble and membrane proteomes.

**Click chemistry and removal of excess reagents**

1. For each sample, add the following reagents (make 'click stock' and add 55uL/sample):
For 22 samples = 11 ML proteome, 110 μL Biotin-Peg3, 220 μL TCEP, 660 μL TBTA, 220μL CuSO4

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|  | Volume added | Final Concen |
| Proteome | 1 mL | 1 mg/mL |
| CuSO4 (50mM stock in water) | 20 μL | 2 mM |
| Biotin-PEG3-azide (ChemPep Inc., cat #271605) | 10 μL | 100 μΜ |
| Tris(2-carboxyethyl) phosphine 1.2mg/500m (TCEP) dal | 20 μL | 1 mM |
| Tris[(1-benzyl-1H-1,2,3-triazol-4- yl)methyl]amine (TBTA, 1.7mM in DMSO-tBuOH (1:4 v/v) | 60 μL |  |

1. Carry out click reaction for 1 hr at room temperature while shaking, or vortex every 15 min.
2. Transfer to a 15 mL conical on ice, add cold MeOH (2 mL) and vortex.
3. Centrifuge at 5000 rpm for 10 min, creating a protein pellet
4. Carefully remove top. Wash pellet with 1:1 MeOH:CHCI3 (1 mL,
5. Remove washings and resuspend pellet in cold MeOH (2 mL) and sonicate, resulting in a cloudy solution. Add cold CHCI3 (0.5 mL).
6. Centrifuge at 5000 rpm for 10 min to pellet protein and remove supernatant
Denature, Reduce and Alkylate

**Denature, Reduce and Alkylate**

1. To each sample, add freshly made 6M urea in DPBS (500 µL), followed up 10uL of 10%SDS (mixture does not have to be clear - clears after incubation)
2. Premix equal volumes of freshly prepared TCEP (200mM in DPBS) and K2CO3 (600 mM in DPBS). 50 µL of this solution added to each sample.
3. Pellet resuspended by sonication and solution incubated for 30 min at 37 C on a shaker.
4. To each sample, 70 µL of a freshly prepared 400mM (in DPBS) iodoacetamide (IAA) solution added. Solution incubated at room temperature protected from light.
5. To each solution, 130 µL of 10% SDS in DPBS added and sample diluted with 5.5 mL DPBS.

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| 6 M Urea | 1.8 g/5 mL (10 Samples) |
| 200 mM TCEP | 57 mg/mL |
| 600 mM K2CO3  | 83 mg/mL |
| 400 mM IAA | 74 mg/mL |

**TMT labeling of protein-enrichments**

1. Perform last 2 washes of beads in 200mM EPPS pH 8
2. Resuspend beads in 200mM EPPS 2M Urea
3. Add normal amount of trypsin
4. Next morning-remove sample from beads (~220 µL)
5. Add dry ACN to 30% final volume (~95 µL)
6. Add 6 ul of respective 10-plex TMT tag (for FFF probes), 3 µL (for FP
probes) to each sample
7. Vortex and incubate at RT for 1 hr-1 h 15 min
8. Add 6 µL of 5% hydroxylamine to each sample, vortex and incubate 15 minutes
9. Add 4 µL formic acid and vortex
10. Dry down to ~100 µL and store at -80c

**Resuspending samples**:

1. Add 400-500ul fresh buffer A (95% Water, 5% ACN, 0.1% Formic) to the first tube
2. Pipette up and down and vortex to resuspend
3. Hard spin in microfuge and transfer to second tube-resuspend, vortex, spin and add to third tube (and so-on and soon until 10th tube is resuspended)
4. Add 200 ul buffer A to the empty tube 1- vortex and hard spin and transfer to tube 2 (repeat vortex and transfer for remaining tubes)
5. Final volume should be ~ 800ul - measure with P1000 pipette and load half onto prepared fat butt ~121