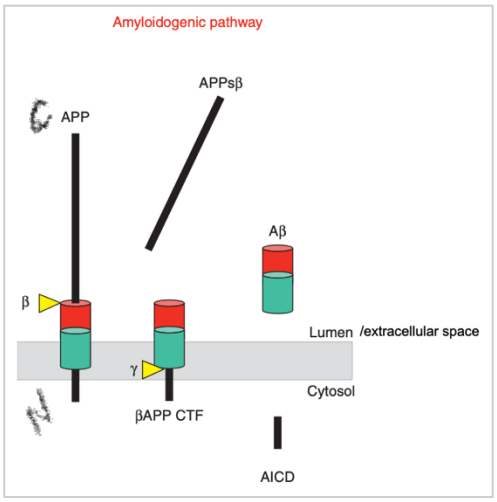
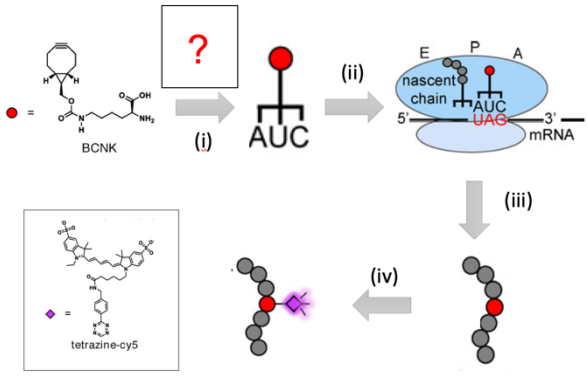
CH-313 (Autumn 2023: Instructor: Prof. Yimon Aye; PhD-student teaching fellow: Song Chen) ANSWER KEY
In-class Problem Set (Lecture 7-8) **There are 3 problems in total but we will only take the best-scoring two!**

Q1. Genetic Code Expansion (GCE) technology & applications. Key pathological hallmarks of Alzheimer's disease is linked to accumulations of insoluble deposits of amyloid beta peptide (Aβ). Figure in box below shows how Aβ is derived: a step-wise proteolytic processing, starting from the β-amyloid precursor protein (APP), which is a large trans-membrane protein (see figure), is catalyzed by two proteases β and γ (at the sites marked by yellow triangles), generating Aβ. These enzymatic processing steps are well studied. However, spatiotemporal dynamics of Aβ generation/APP processing *in cellular context* are less well understood. You decide to use GCE technology to track APP proteolytic processing and trafficking of resulting Aβ and also AICD (APP intracellular domain) (see figure).



(i) Figure below is a schematic illustration of concepts underlying GCE. List the component(s) missing in the box marked by ?
Plasmids encoding **orthogonal tRNA** and **orthogonal amino-acyl tRNA synthetase (aaRS)**

NOTE/FYI: BCNK is cell permeable. In class, we came across Cu(I)-catalyzed [3+2] cycloaddition/Click coupling using a terminal alkyne. Step (iv) within this figure below shows another biocompatible chemical reaction, cyclo-octyne-ring-strain-promoted [4+2] Diels-Alder cycloaddition with tetrazine:



(ii) In the boxed figure shown directly above, indicate where you would
(a) genetically fuse GFP protein (i.e., at the N or C terminus as shown), and
(b) encode TAG (amber suppressor codon), in your APP protein, such that ultimately you can track, by imaging assays, both AICD and Aβ *both before and after* cleavages by *both* proteases. **NOTE:** N and C terminus of APP is written out in the figure. You want to put TAG in-between the two protease cleavage sites shown by two yellow triangles

(iii) You "transfect" the cells (i.e., deliver exogenous DNAs so that you can express desired biomolecules in live cells) with plasmid-DNA encoding your APP, that houses the TAG-amber suppresor codon at a defined site as well as GFP fusion as you decided in **(ii)**. You also co-transfect the same cells with other necessary plasmid(s) that enable GCE (related to ? in **(i)** above). At a specific time, you incubate your cells with BCNK (see **(i)** above). After waiting for ~6 hours (the time you know it takes for BCNK incorporation), you fix/permeabilize the cells and treat with tetrazine-Cy5 (see "Step (iv)" in **(i)** above). You then use excitation channels for GFP and Cy5 to acquire cell images.

- Before any protease cleavage: where should your GFP and Cy5 signal be localized? Choose correct word: "cytosol" vs. "extracellular space" in each case.
  GFP signal: cytosol Cy5 signal: extracellular

  Without doing any additional experiment, but in the same images above, what else would you look for, to ensure that both GFP fusion and GCE are functionally operating as expected? Provide your answer in a single word:

  Co-localization of GFP and Cy5 signal on the membrane

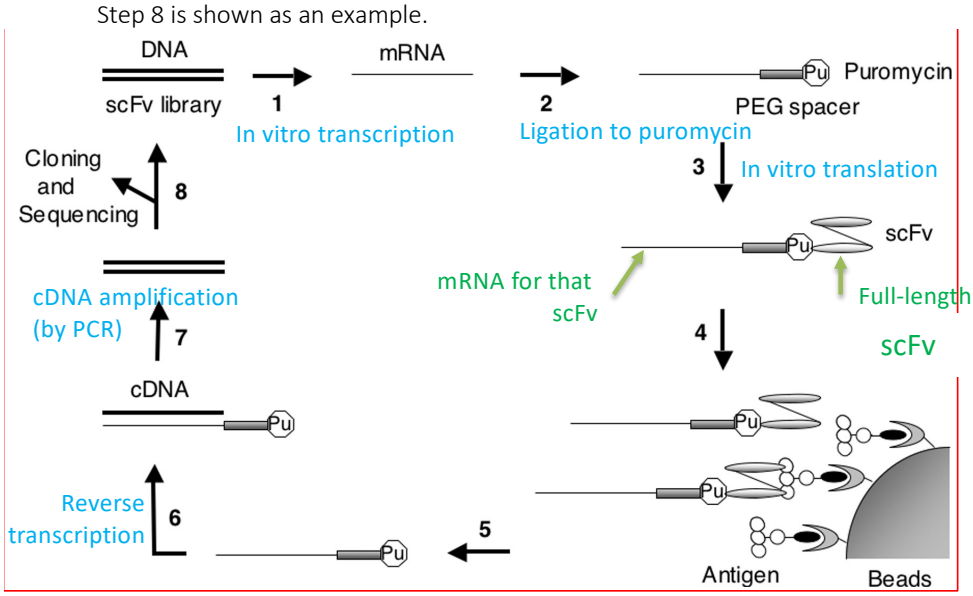
- After full APP-processing by both proteases: where should your GFP and Cy5 signal be localized? Choose correct word: "cytosol" vs. "extracellular space" in each case.
  GFP signal: cytosol Cy5 signal: extracellular

(iv) It is well known that extracellular Aβ can be internalized (into the cell). However, it has also been hypothesized that extracellular Aβ is cleared before intracellular Aβ. In one single phrase, how would you extend your assay above to test this hypothesis? Time course assay (e.g., treat cells with BCNK and incubate for several time periods and analyze at each time-point)

(v) List (a) necessary control(s) required to solidify your conclusions/interpretations from your experiments above.
-- Transfect cells with all necessary components of GCE but leave out BCNK;
-- Use "control" plasmid encoding APP but without introducing TAG (amber suppressor codon)
-- Use empty plasmid that does not encode either tRNA or aaRS, but keep all the rest of the conditions the same including BCNK treatment.
IMPORTANT NOTE: the key take-home here is that to regard any condition as a control, you must change only one parameter at a time. Otherwise you lose the comparison of what causes the observed change..

Q2. mRNA display technology and applications. The workflow below shows in vitro evolution of single-chain Fv antibody fragments (scFv) using mRNA display.

(i) Indicate processes underlying each of the Steps, 1 to 8 within the space available in figure. ("Pu" is puromycin). Step 8 is shown as an example.



Step 4: Binding the mRNA-displayed ScFv library to antigen-immobilized beads and washing away the unbound.

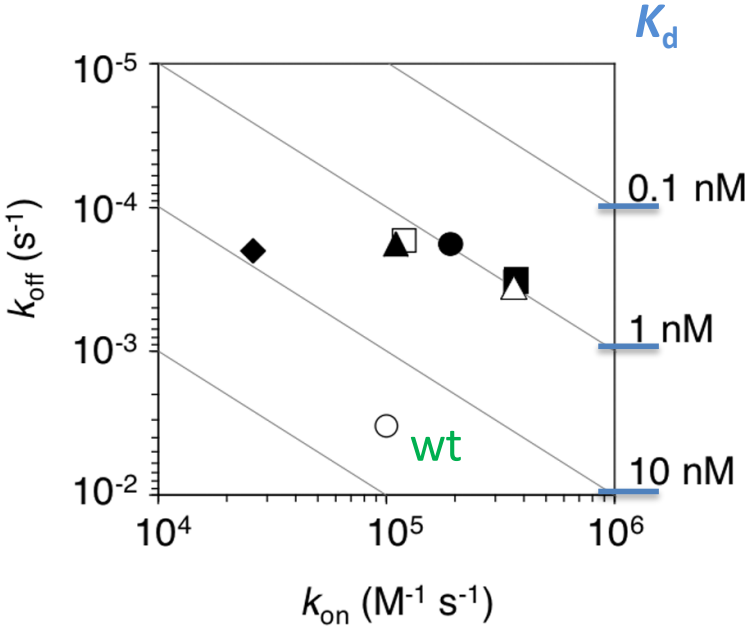
Step 5: Elution of the bound (by protease digestion – e.g., poteinase K treatment)

Step 8: DNA that encodes the specific eluted scFv can be fed forward to the next round of selection (or can be analyzed by sequencing etc.)

(ii) 6 purified scFv mutants (marked in cricles, diamond, and square on the plot) were preliminarily selected and kinetic parameters for antigen binding were evaluated against wild-type (wt) scFv (marked in green on the plot).

(a) What specific kinetic parameter is significantly altered in *all* mutants discovered from mRNA display, compared to wt scFv? Compared to WT, k_{off} values of all mutants are smaller, meaning that they dissociate from antigen more slowly than WT ScFv

(b) Is this outcome consistent with the experimental setup for enrichment protocol shown? Answer Yes or No, and provide a concise reason (1-2 sentences only).
Because pulldown experiments have to implement extensive washing steps, these experiments are often dominated by koff, Thus it is not unusual to find binders with small koff values since such binders will always be favored (i.e., retain on the beads better during the wash steps)



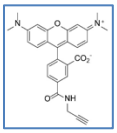
(c) What do the gray diagonal lines represent? And state the values on the 3 intersection points (marked in red boxes) on the right-hand Y-axis. (**Hint:** think about what you can derive from k_{on} and k_{off}) – K_d (see answers in plot above)

Q3. Global incorporation of UAAs (e.g., SILAC-BONCAT) and applications.

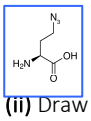
Bacteria use a process of chemical communication called "quorum sensing" to assess their population density and to change their behavior in response to fluctuations in cell number and species composition of the community. In this problem, you set out to identify the quorum-sensing-regulated proteome in the model organism *Vibrio harveyi* by bio-orthogonal non-canonical amino acid tagging (BONCAT). A type of *V. harveyi* strain you used responds precisely and exclusively to exogenously supplied small-molecule AI-1 that induces quorum sensing.

To start off, following addition of AI-1 to the cells, you monitor changes in protein synthesis in 10-min intervals, by combining BONCAT with SILAC technique. In your chemical inventory, you have the following chemicals available (all natural and unnatural amino acids are in natural stereoconfiguration where applicable):

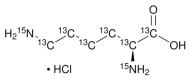
- azidohomoalanine (Aha)
- TAMRA-alkyne (note: TAMRA is a small-molecule red fluorescent dye) (structure shown on right→)
- Lysine (also known as "light"/unlabeled lysine)
- $^{13}C_6$ $^{15}N_2$ Lysine (aka "heavy"/labeled lysine)



(i) Draw out the chemical structure of Aha. You may show the correct stereochemistry. (Hint: organic chemistry nomenclature reminder: "homo" is for one carbon extension; for instance, H2C=CHCH$_2$Cl is allyl chloride, and H$_2$C=CHCH$_2$CH$_2$Cl is homoallyl chloride)



(ii) Draw out the chemical structure of $^{13}C_6$ $^{15}N_2$ Lysine. You may show the correct stereochemistry.



note: this is sold as an HCl-salt commercially but HCl indication does not count for any point in the answer key

(iii) In your drawing above in (ii), mark the isotopically-labeled carbon and nitrogens as open and closed circles, respectively.

See above

(iv) You first wanted to ensure that

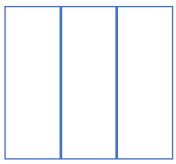
a)-- a 10-min Aha pulse is sufficient for incorporation of this uaa.

b)-- the use of chloramphenicol (Cam) treatment is sufficient to halt new protein synthesis after the 10-min pulse.

Suggest conditions A, B, and C that would help ensure the two points (a & b) above, *and absoultely those two points alone*, using a gel-based analysis. **You're limited to 3 lanes only: i.e., 3-different experimental conditions. Hint: Two of the conditions should be relevant negative controls such that you can derive meaningful outcomes from your experiments. [Key note: *Aha is known to be incorporated into proteins in competition with Methionine (Met)*]**

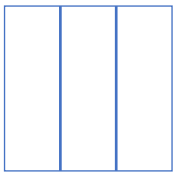
Gel result upon excitation of TAMRA dye channel on the imager (i.e., in-gel fluorescence analysis):

A B C

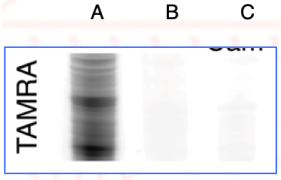


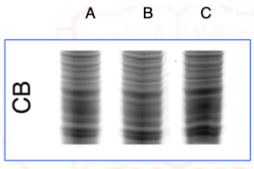
The same gel after Coomassie-dye staining:

A B C



A = Aha; B = Met; C = Aha + Cam





In all conditions, you treated the cells, lyse the cells, execute Click coupling with TAMRA-alkyne, and run SDS-PAGE.

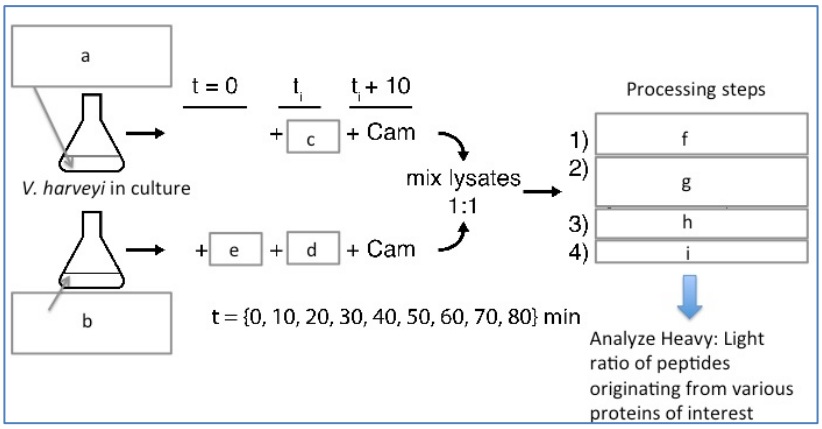
Condition A involves treatment of the cells with _____Aha_____

Condition B involves treatment of the cells with _____Met_____

Condition C involves treatment of the cells with __Aha together with protein synthesis inhibitor Cam

(v) Various Lux genes are encoded by the *lux* operon in *V. harveyi* and are central to regulation of quorum sensing. (note: 'operon' means a group of genes regulated under the same promoter). But at the outset of your work, it was unknown how the translational temporal dynamics of Lux genes alter upon activation of quorum sensing. You next set up the combined SILAC-BONCAT approach for quantifying differences in protein translation in response to treatment with a small molecule that upregulates quorum sensing. Fill in the missing blanks in the workflow shown below. [Note: addition of chloramphenicol (Cam) halts protein synthesis].

a
b
c
d
e
f
g
h
i



a "Light" $^{12}C_6{}^{14}N_2$ L-Lys

b "Heavy" $^{13}C_6{}^{15}N_2$ L-Lys

c Aha

d Aha

e AI-1

f Ligate affinity tag (Click)

g enrich newly synthesized proteins

h LysC (or protease) digest

i LC-MS/MS