Announcements

- Chapter 8 Week 1 by EOD Friday
- Chapter 8 Lab report template released Wed, Feb 11
- Chapter 7 Lab report grades will be returned by the Monday after the B5 section turns their reports in
- Lab feedback

Announcements

- Dr. Tolan here later to discuss Creativity Project

Chapter 8AB: In vitro transcription and translation

Objectives

- To use an in vitro transcription and translation system to synthesize proteins from genes cloned into a plasmid.
- Introduction to fluorescence

Procedures

- Use a **combined RNA polymerase transcription system** and a **wheat germ lysate-translation system** to ***express and synthesize proteins*** from cloned genes
- Use SDS-PAGE analysis to analyze protein expression

Transcription and Protein Synthesis

Week 1: Add DNA template and perform transcription/translation using TNT® Wheat Germ Extract.

Week 2: Separate translation products by SDS-PAGE

Fluorescent Imaging

Central dogma of molecular biology

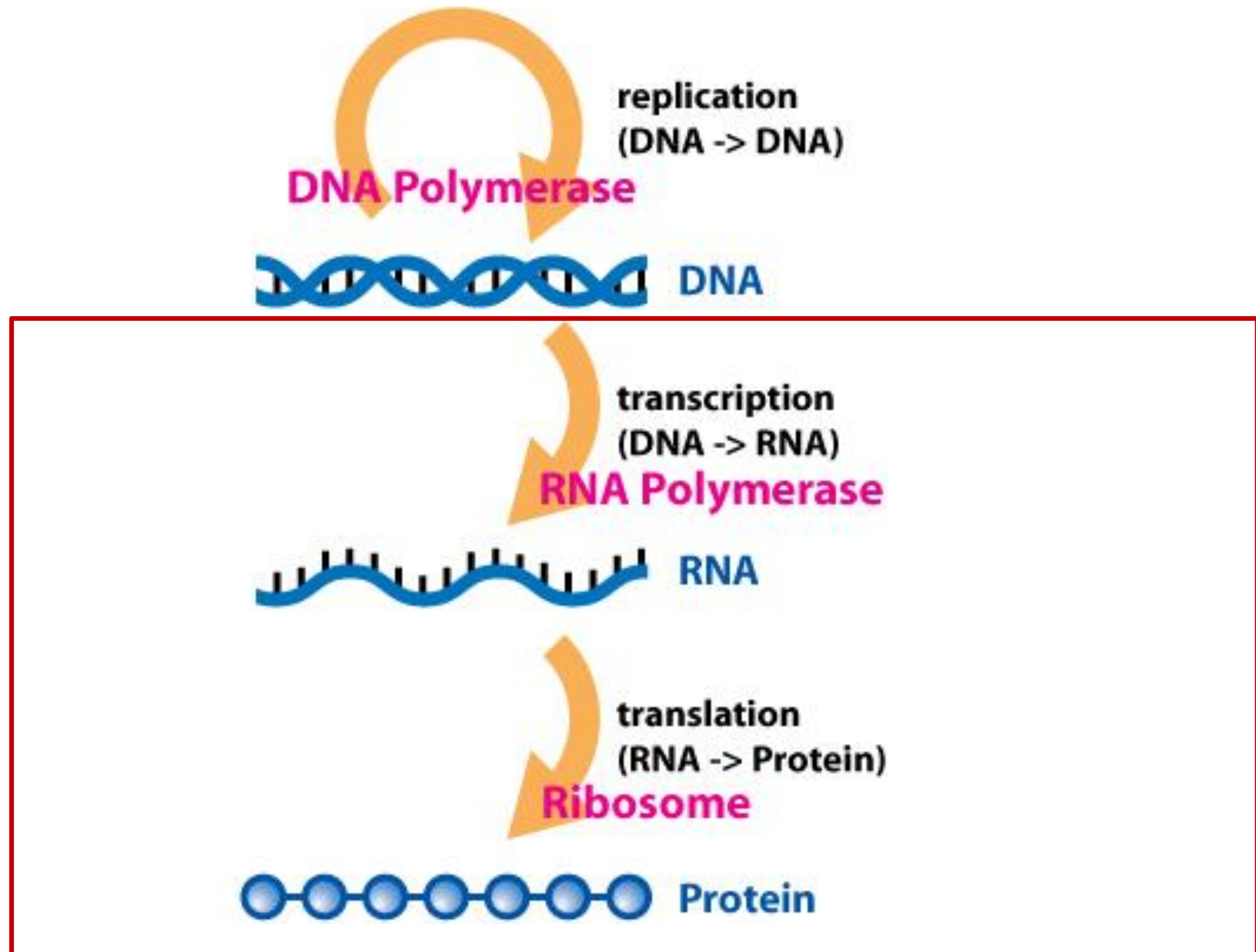

Photo: Wikipedia (2008).

In vitro transcription/translation

- DNA systems: coupled transcription/translation
- RNA systems: translation only

- Chapter 8 will use a ***DNA system*** (plasmid DNA > RNA > Protein)
  - Components in system include:
    - SP6 polymerase
    - NTPs (ATP, GTP, CTP, UTP)
    - Amino acids
    - Wheat Germ Extract
      - Ribosome and other translation factors
      - tRNAs
      - GTP (source of translation energy)
      - Fluortect $Green_{Lys}$
  - Students will supply the plasmid DNA

Recall from Chapter 6…

- Isolated pGEM3-Rel and pGEM4-Rel from *E. coli* using mini-prep
- Each plasmid contains:
  - **SP6 promoter**
  - **Target gene - *Rel***
  - **Ampicillin resistance gene**
  - **Origin of replication**
  - **Restriction enzyme recognition sites**
- Transcription only starts at sites with specific promoter sequences
- Incorporated just upstream of cloning sites of any opening reading frame

Plasmid maps Figure 6-5, p. 209

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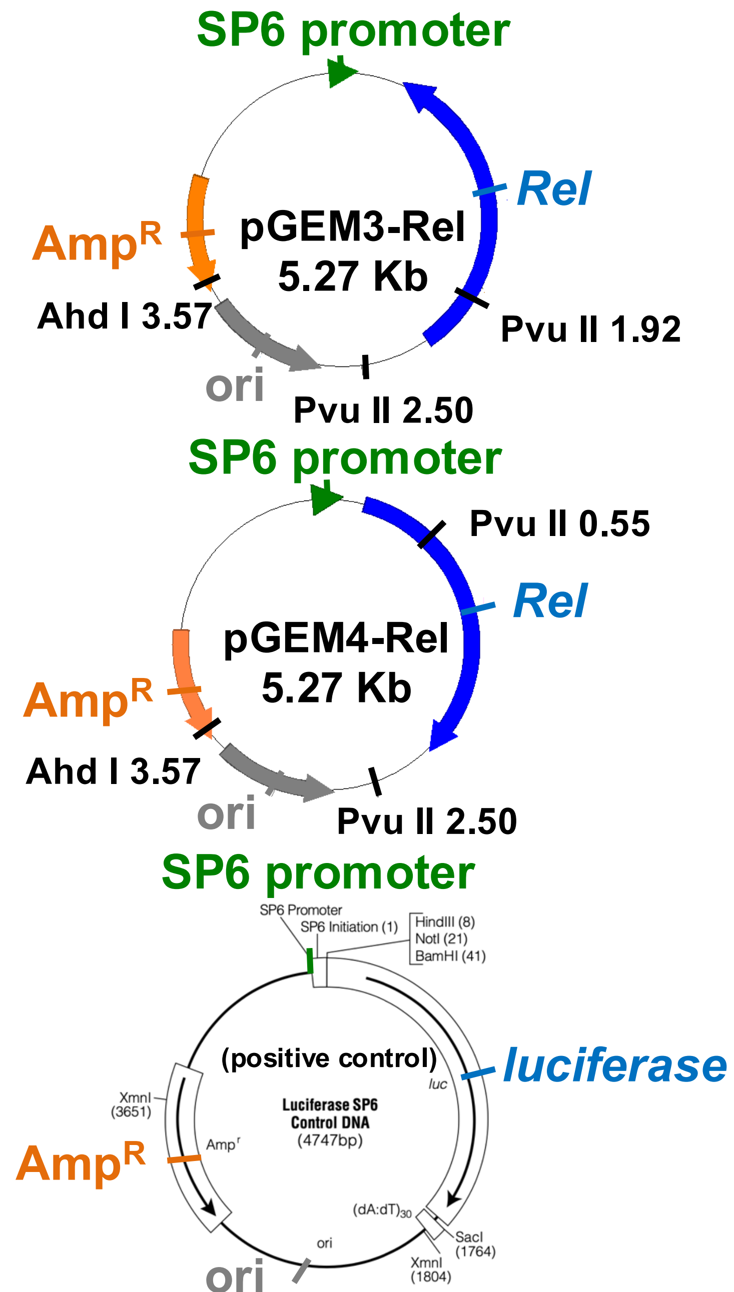
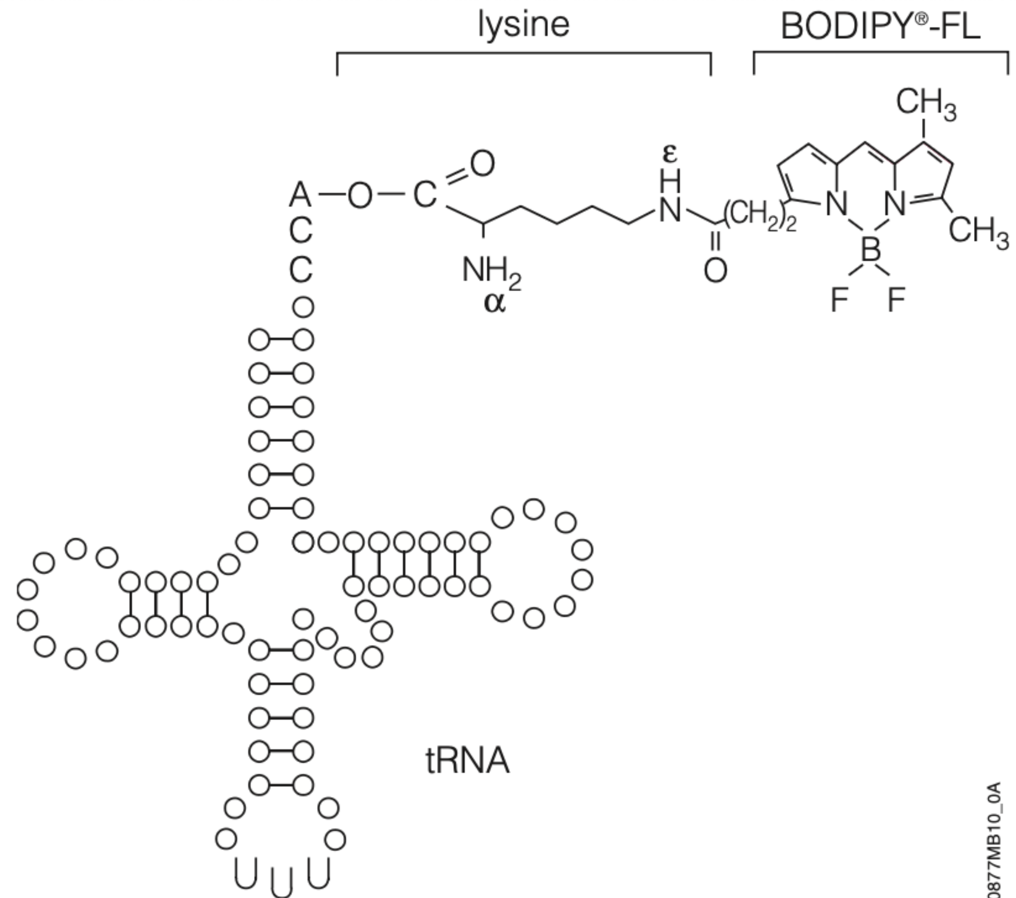


Plasmid maps Figure 6-5, p. 209

How will we know if it worked?

Use a fluorescently labeled amino acid!



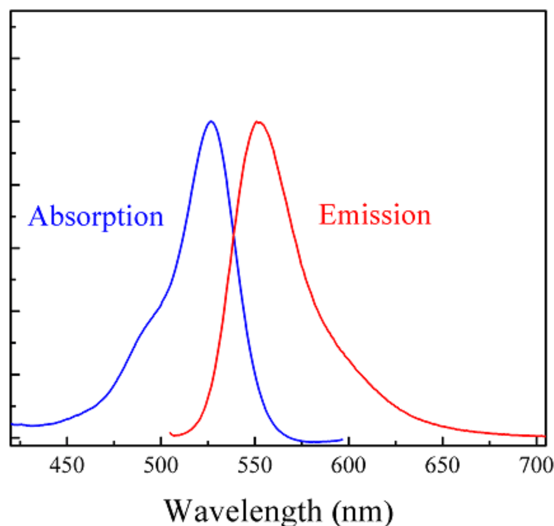
Fluorescence spectroscopy

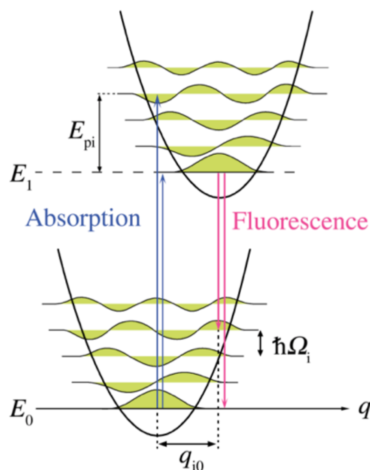
- Molecules that are capable of fluorescence are known as fluorophores
- Fluorophores absorb light energy at particular wavelengths and energy levels (absorption) and reach an excited state (excitation)

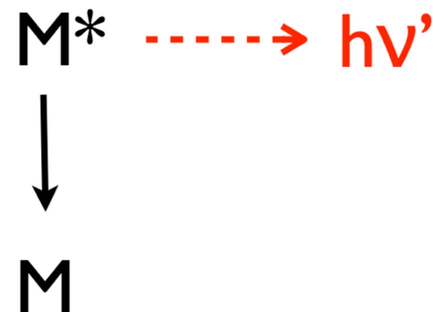
- It then emits light at longer wavelengths at a lower energy level (emission)

- Net reaction: $M + h\nu \rightarrow M^* \rightarrow M + h\nu'$

$h\nu > h\nu'$

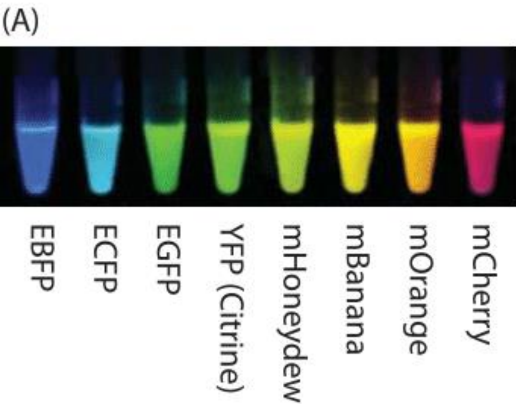






https://www.chem.uci.edu/~unicorn/M3LC/handouts/Week2/SpectroscopyHandout.pdf

Molecules and materials can be intrinsically fluorescent or synthesized



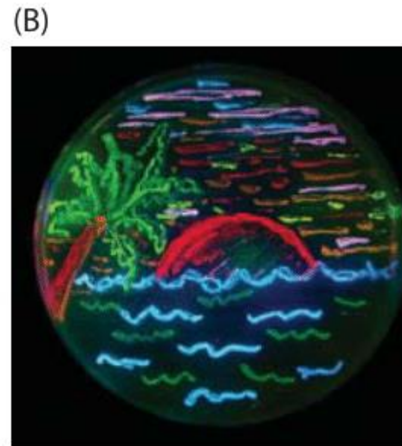
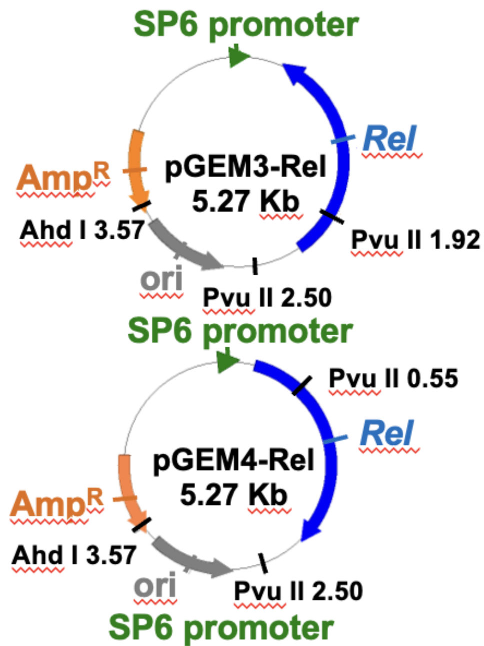


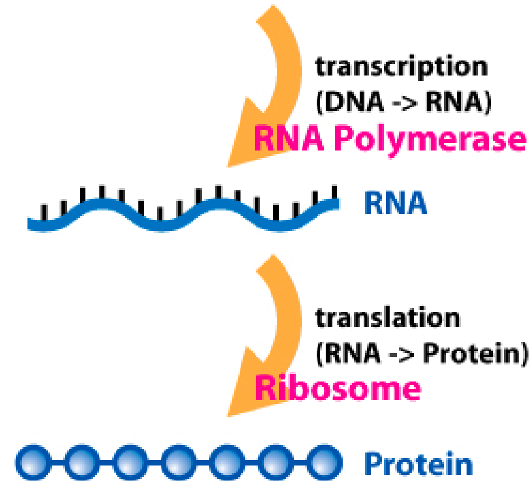
Illustration of some of the palette of fluorescent proteins that has revolutionized cell biology.
(A) Fluorescent proteins spanning a range of excitation and emission wavelengths. (B) Illustration of a petri dish with bacteria harboring eight different colors of fluorescent protein and used to “paint” an idyllic beach scene. (Adapted from: R. Y. Tsien, Nobel lecture, Integr. Biol.,2, 77-93, (2010).)

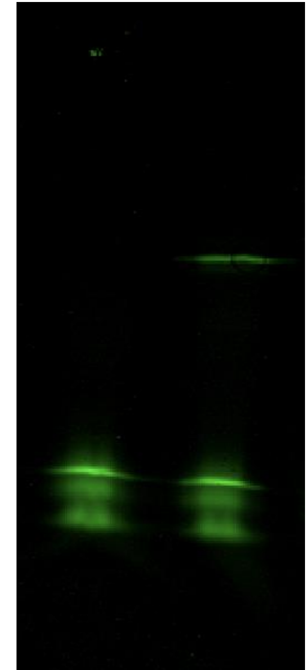
Examples of fluorescent molecules

- Amino acids (Trp, Phe, Tyr)
- Base pair derivatives (2-AP, 3-MI, 6-MI, 6-MAP, pyrrolo-C, tC)
- Chlorophylls
- Fluorescent Proteins (FPs)
- Organic dyes (fluorescein, rhodamine, N-aminocoumarins and derivatives of these)
- Rare earth elements (lanthanides)
- Quantum dots
- And many more…

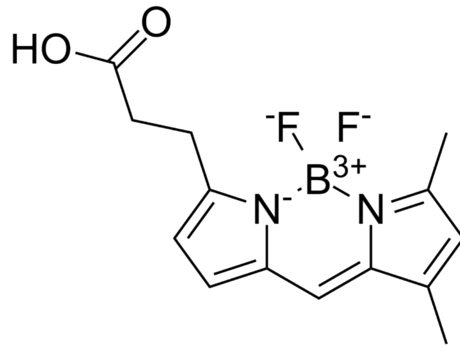
Basic Flowchart of Methods

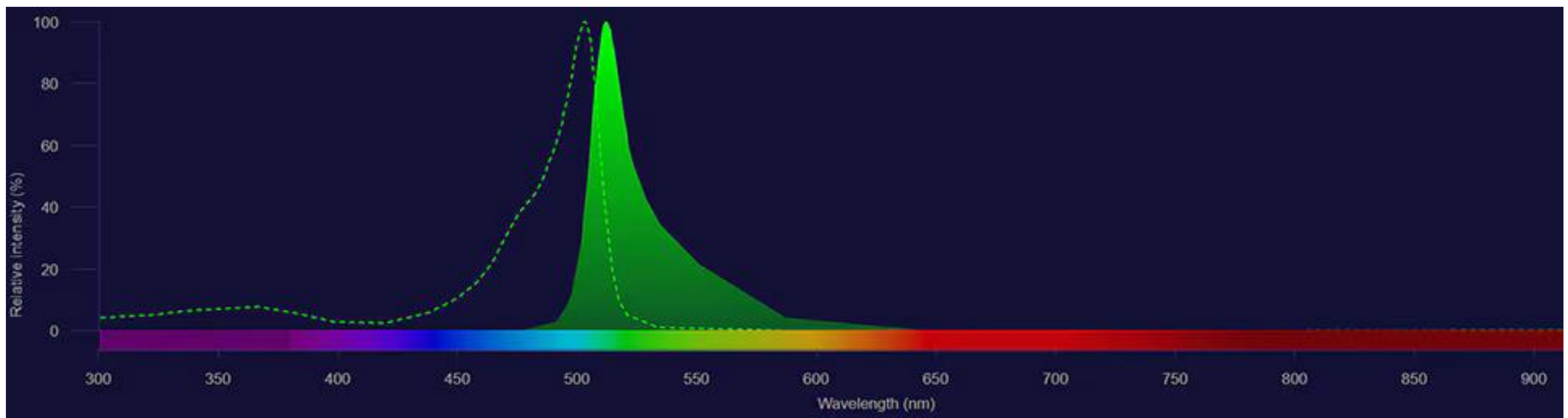






Intro to BODIPY-FL





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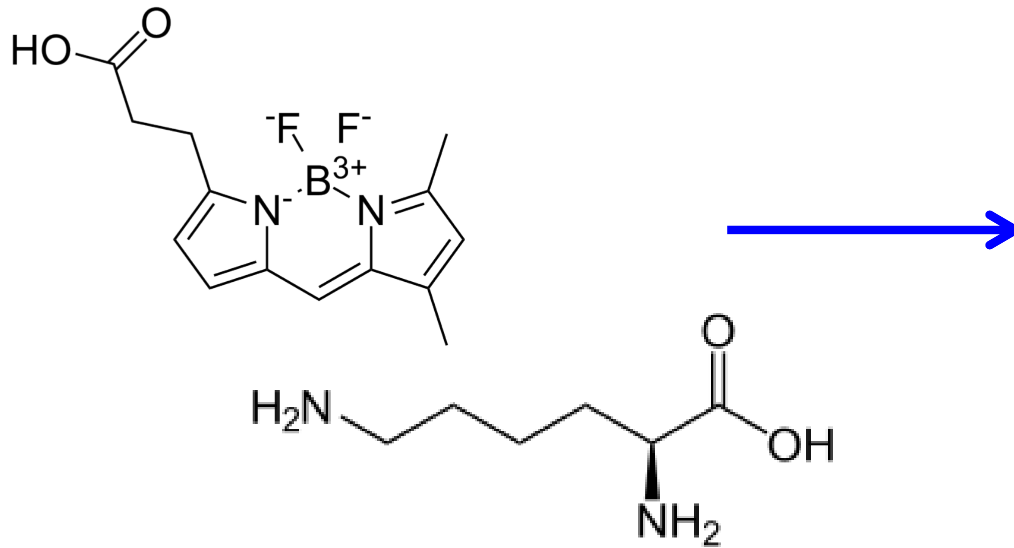

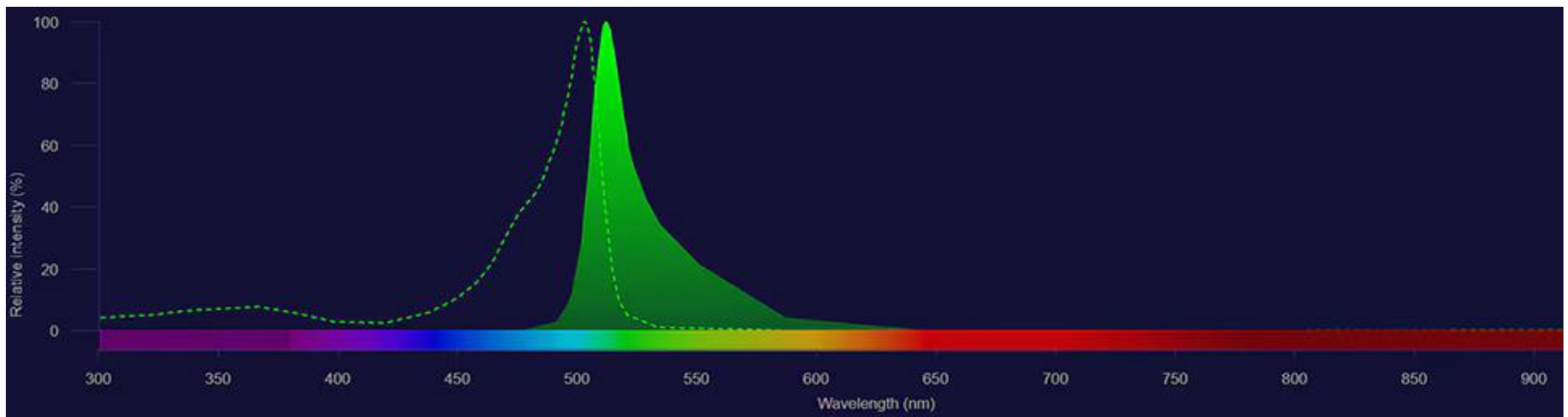

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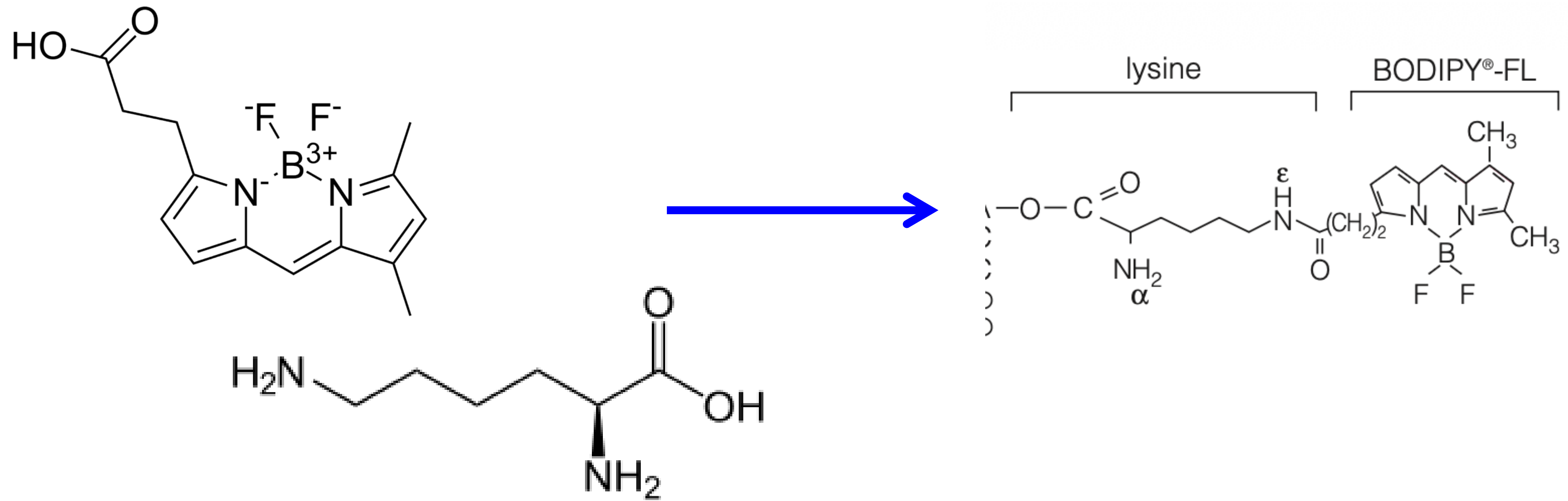

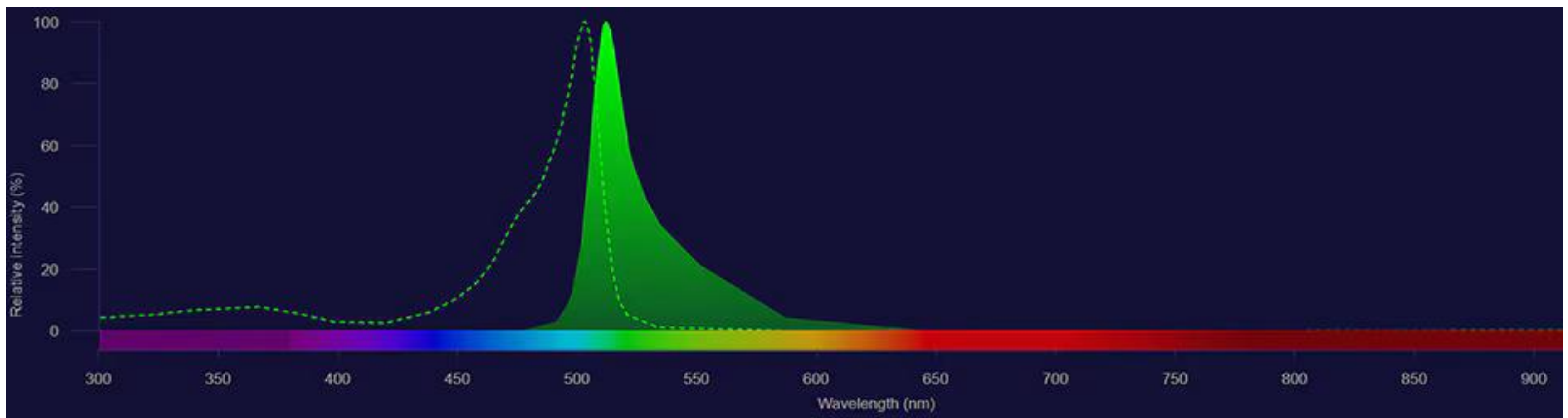

Nothing is forever

- Fluorophores can only fluoresce a limited number of times
- If they are under room light for too long, you won't be able to see them on the gel.
- When samples are in the ice bucket, cover with lid.
- When covering with the bucket lid is not practical, wrap in aluminum foil.

Properties of RNases

- RNA is easily degraded by ribonucleases, RNases
- These enzymes are on your clothes, skin, saliva
- Very important to work with gloves
- You can inactivate RNases by sterilizing solutions at high temperatures in the presence of DEPC, or by using chemical modifications

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We want to make RNA transcripts, be careful of RNase contamination!

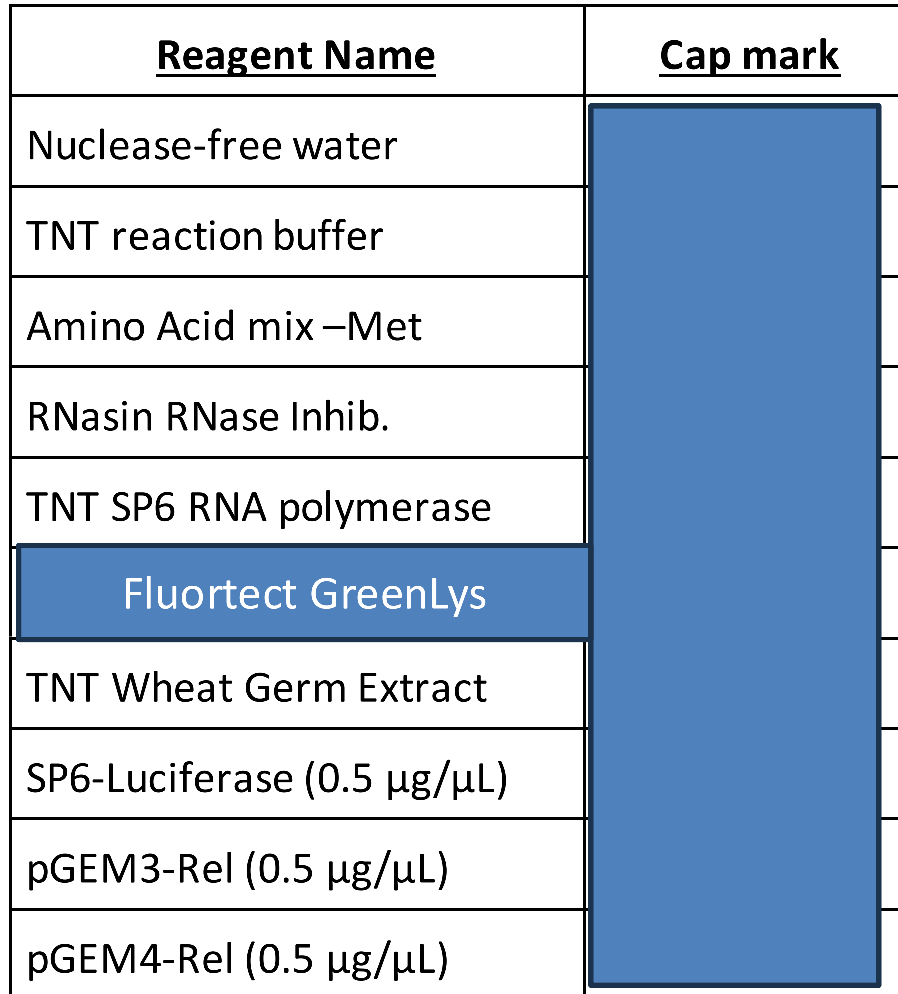
Chapter 8AB: Procedure

- Use gloves at **all times** to prevent RNase contamination
- We will provide sterile/RNase-free pipet tips, centrifuge tubes, etc. **Please keep them that way!!!**
- You will be pipetting **very small volumes!**
  - ➢ We should have some P-2 pipets available **(0.2 – 2 μL)**

Chapter 8AB: Procedure

- Tips for working with small volumes:
  - Spin down samples in tabletop centrifuge
  - Pipette samples as little as possible
  - Pipette larger volumes into smaller volumes
  - Check pipette tips

Chapter 8AB: Reagent List (example)– make your own! It will be different from this



Reagent Name	Cap mark
Nuclease-free water	
TNT reaction buffer	
Amino Acid mix –Met	
RNasin RNase Inhib.	
TNT SP6 RNA polymerase	
Fluortect GreenLys	
TNT Wheat Germ Extract	
SP6-Luciferase (0.5 μg/μL)	
pGEM3-Rel (0.5 μg/μL)	
pGEM4-Rel (0.5 μg/μL)	

Chapter 8AB: Procedure

A. Transcription/Translation:

- Work together with a second group for the first part
- Thaw reagents
- Set up cocktail for 4 reactions according to protocol

→ Prepare mastermix in tube pre-aliquoted with your fluorescent lysine

- Remove 5 µL x 2 for zero-time point control reactions
- Aliquot cocktail, 19 µL / reaction and add 1 µL DNA to each
  - pGEM3-Rel
  - pGEM4-Rel
  - Positive control – DNA template encoding luciferase (SP6-luciferase)
  - Negative control – No DNA (nuclease-free water)

Chapter 8AB: Procedure

A. Transcription/Translation:

- Incubate all samples at **30 ℃ for 90 min**
- Stop reactions on ice
- Split reactions – 10 µL/group
- Each group works separately from now on
- Prep samples for SDS-Page gel
- Store samples at -20 ℃ until next lab

Chapter 8AB: Procedure

- You will be supplied with the positive control plasmid "SP6-Luciferase" containing the ***luciferase*** gene
  - This plasmid has the optimal coding sequence for the SP6 promoter
  - Lots of RNA > lots of protein > a lot of fluorescence
- You may choose to use your own A & B plasmids (check concentration and determine how much to use)
- We can also supply you with A & B plasmids

Chapter 8AB requirements

- Please make sure to submit your Pre-Lab to GradeScope *before* your lab section
- Include in your procedures:
  - a flowchart for the transcription/translation protocol
  - table for the code for reagent tubes
- Please submit your in-lab data collection assignment to Gradescope *by the end of the day after* your lab section

Chapter 8AB

Before the lab period, you should have:

- ✓ Completed your Pre-lab Write-up and submit on Gradescope
  - ✓ Title, purpose and procedures
  - ✓ Remember to include:

At the end of lab, you should have:

- ✓ Performed transcription/translation on 4 samples
- ✓ Saved SDS-PAGE samples for next week (the bulk of the radioactivity!). Be sure to label well.

Questions?

In-class activity

&

Discussion Quiz