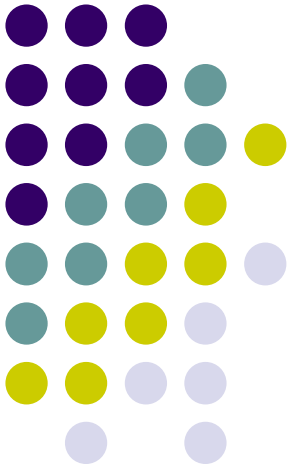
Moc/Bio and Nano/Micro

Lee and Stowell

Moc/Bio-Lecture 5

Experimental Manipulation of Biomolecules

- **DNA amplification and mutation**
- **DNA building blocks**
- **Catalytic antibodies**
- **SELEX and RNA aptamers**
- **Phage display selection**
- **Tagged proteins**
- **Lipids**

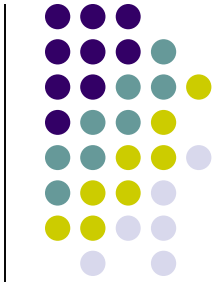


Reading material

- http://www.ncbi.nlm.nih.gov/books/NBK21541/
- http://www.ncbi.nlm.nih.gov/books/NBK21712/
- http://www.ncbi.nlm.nih.gov/books/NBK21733/
- http://www.ncbi.nlm.nih.gov/books/NBK21654/
- http://www.ncbi.nlm.nih.gov/books/NBK21498/
- (DNA melting calculator)

http://www.promega.com/techserv/tools/biomath/calc11.htm

Key concepts of mutation



- Mutation is the process whereby a gene (DNA sequence) changes.
- Mutations can occur spontaneously via several mechanisms
  - DNA damage
  - Errors in replication
- Mutations can be introduced experimentally
- Mutagens increase frequency or rate or mutagenesis in a semi-random manner
- Many biological repair mechanisms eliminate alterations of the DNA
- Mutations can lead to loss of function or give rise to a new function or alter expression.

Remember the genetic code DNA to RNA to Protein

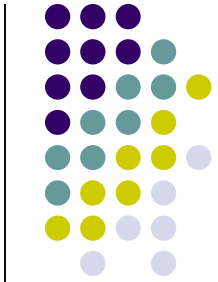
GCA GCC GCG GCU	AGA AGG CGA CGC CGG CGU	GAC GAU	AAC AAU	UGC UGU	GAA GAG	CAA CAG	GGA GGC GGG GGU	CAC CAU	AUA AUC AUU	
Ala	Arg	Asp	Asn	Cys	Glu	Gln	Gly	His	Ile	
A	R	D	N	C	E	Q	G	H	I	
UUA UUG CUA CUC CUG CUU	AAA AAG	AUG	UUC UUU	CCA CCC CCG CCU	AGC AGU UCA UCC UCG UCU	ACA ACC ACG ACU	UGG	UAC UAU	GUA GUC GUG GUU	UAA UAG UGA
Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val	stop
L	K	M	F	P	S	T	W	Y	V	

Figure 6–50. Molecular Biology of the Cell, 4th Edition.

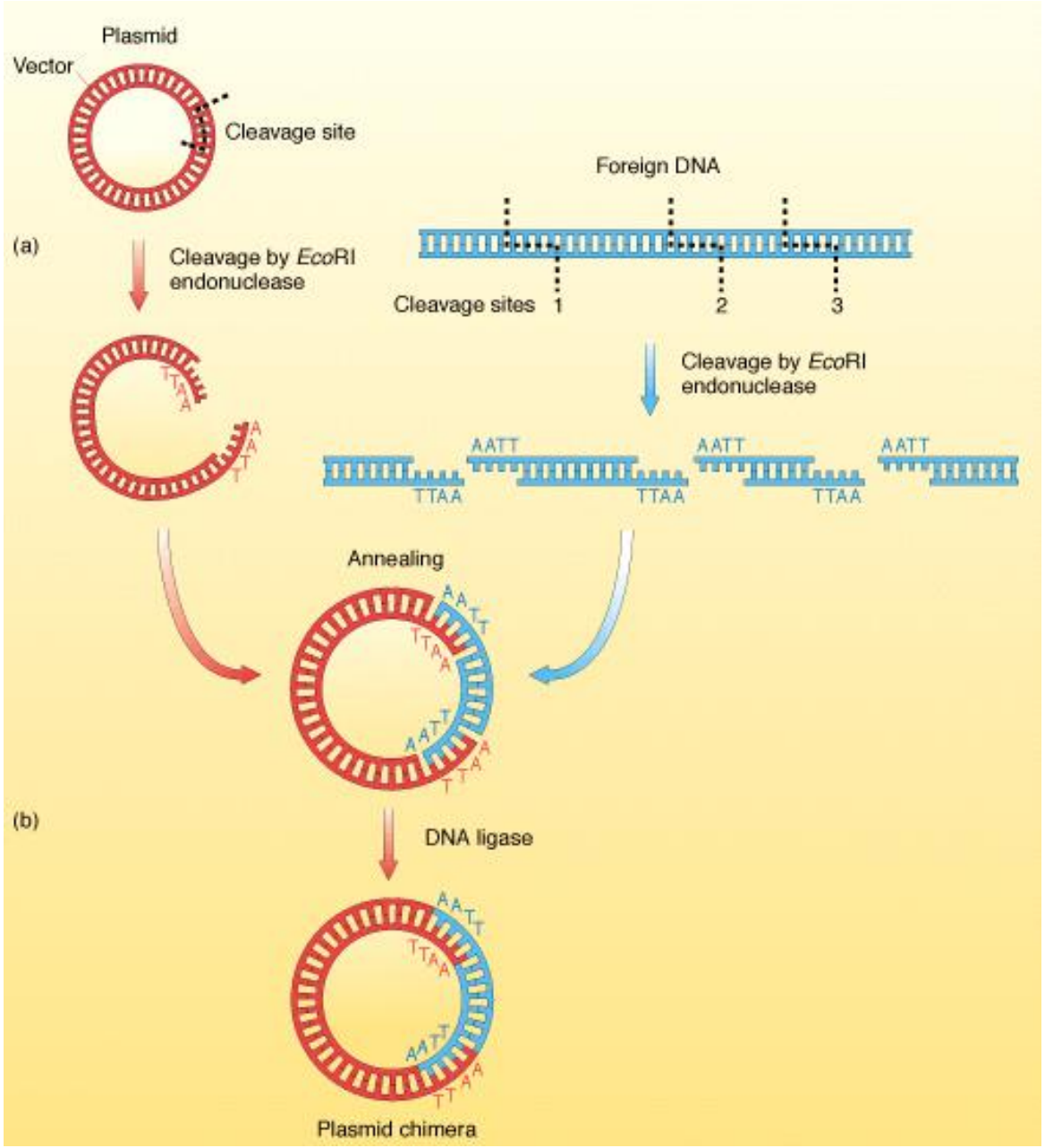
Experimental mutagenesis

- Recombinant DNA technology
- Restriction enzymes
- Vectors and libraries
- PCR techniques
- Homologous recombination
  - Introduce new genes into an organism (gene therapy)

Key concepts



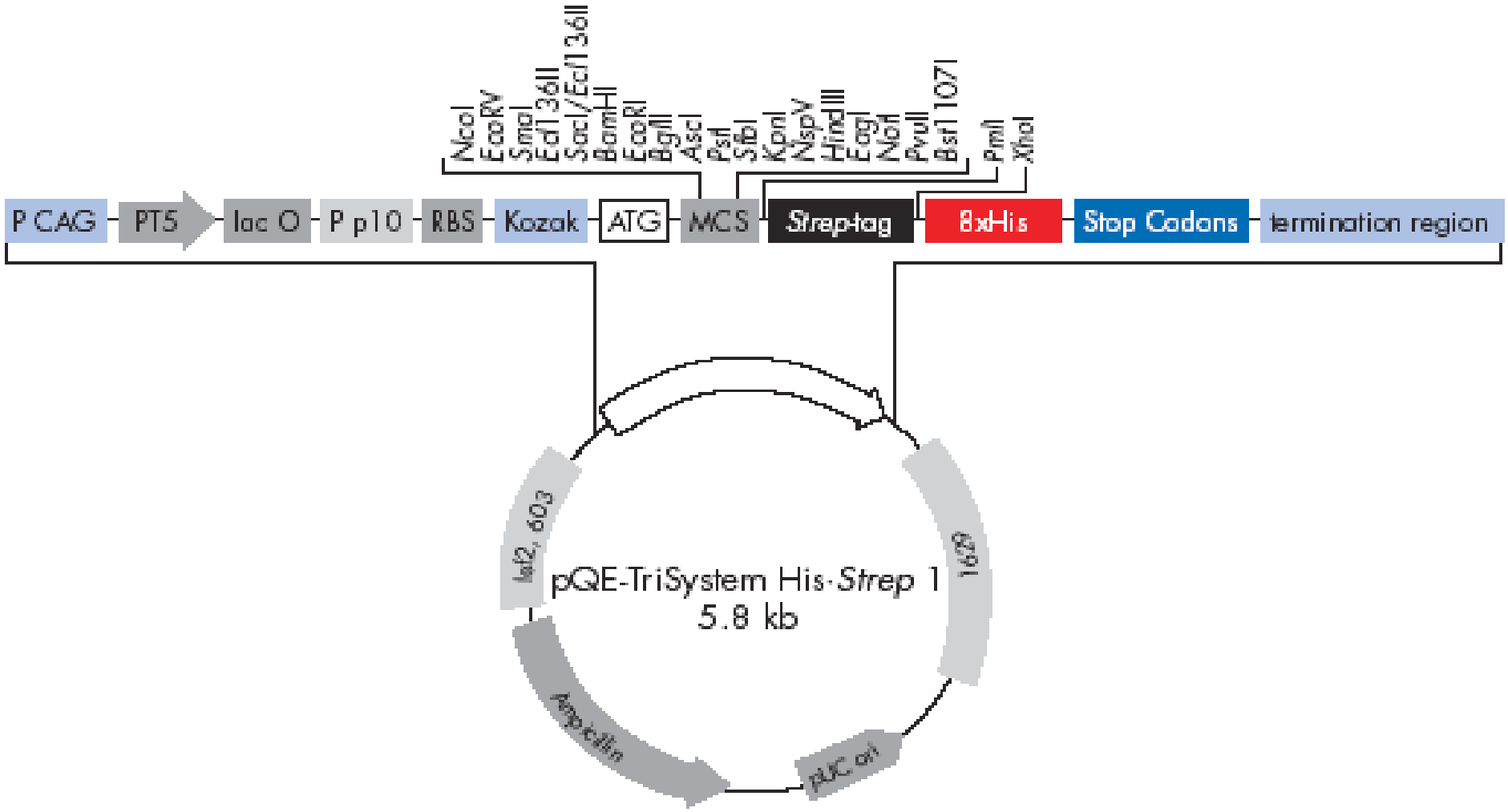
- Recombinant DNA is made by splicing “foreign” DNA fragments into “host” DNA
- Vectors can be used for replication of “foreign” DNA and expression of target proteins
- Restriction enzymes cut DNA at sequence specific sites
- “Foreign” DNA with identical restriction cuts can be specifically ligated into the host DNA
- PCR can be used to amplify and mutate DNA in a design specific manner.
- Homologous recombination can create stably integrated DNA fragments


Plasmid
Vector
Cleavage site
Foreign DNA
(a)
Cleavage by EcoRI endonuclease
Cleavage sites 1 2 3
Cleavage by EcoRI endonuclease
AATT
TTAA
Annealing
(b)
DNA ligase
Plasmid chimera

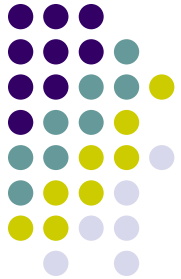
Enzyme	Organism from which derived	Target sequence (cut at *) 5' -->3'
Ava I	Anabaena variabilis	C* C/T C G A/G G
Bam HI	Bacillus amyloliquefaciens	G* G A T C C
Bgl II	Bacillus globigii	A* G A T C T
Eco RI	Escherichia coli RY 13	G* A A T T C
Eco RII	Escherichia coli R245	* C C A/T G G
Hha I	Haemophilus haemolyticus	G C G * C
Hpa I	Haemophilus parainflenzae	G T T * A A C
Mbo I	Moraxella bovis	*G A T C
Pst I	Providencia stuartii	C T G C A * G
Sma I	Serratia marcescens	C C C * G G G
SstI	Streptomyces stanford	G A G C T * C
Sal I	Streptomyces albus G	G * T C G A C

- http://www.firstmarket.com/cutter/cut2.html

An expression vector



PCR amplification and mutagenesis Key concepts



- Primers must be designed for optimal melting temp i.e. not too low and not too high.
- The use of hyperthermophile polymerases
  - Different polymerases have different error rates
  - Fractional errors of 10^{-6} to 10^{-5} per cycle
- Can readily make changes in target DNA
- Fast and inexpensive

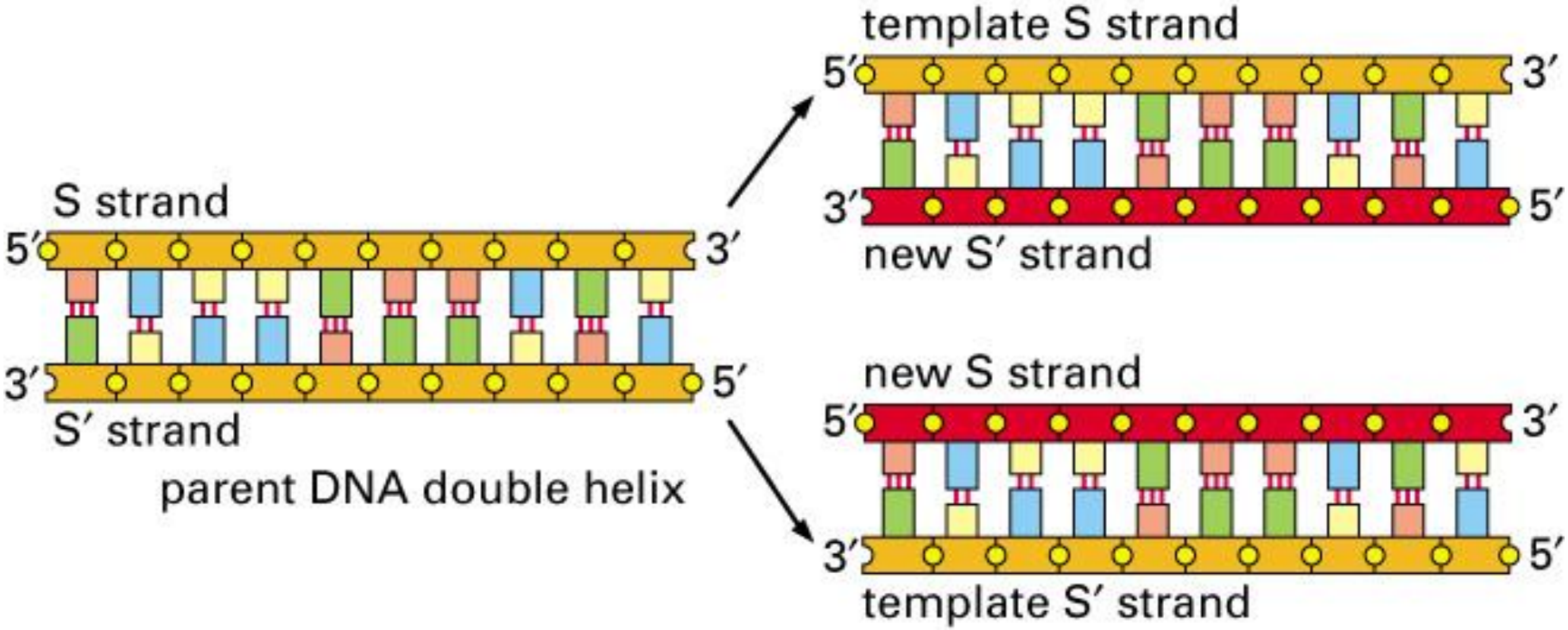


Figure 5–2. Molecular Biology of the Cell, 4th Edition.

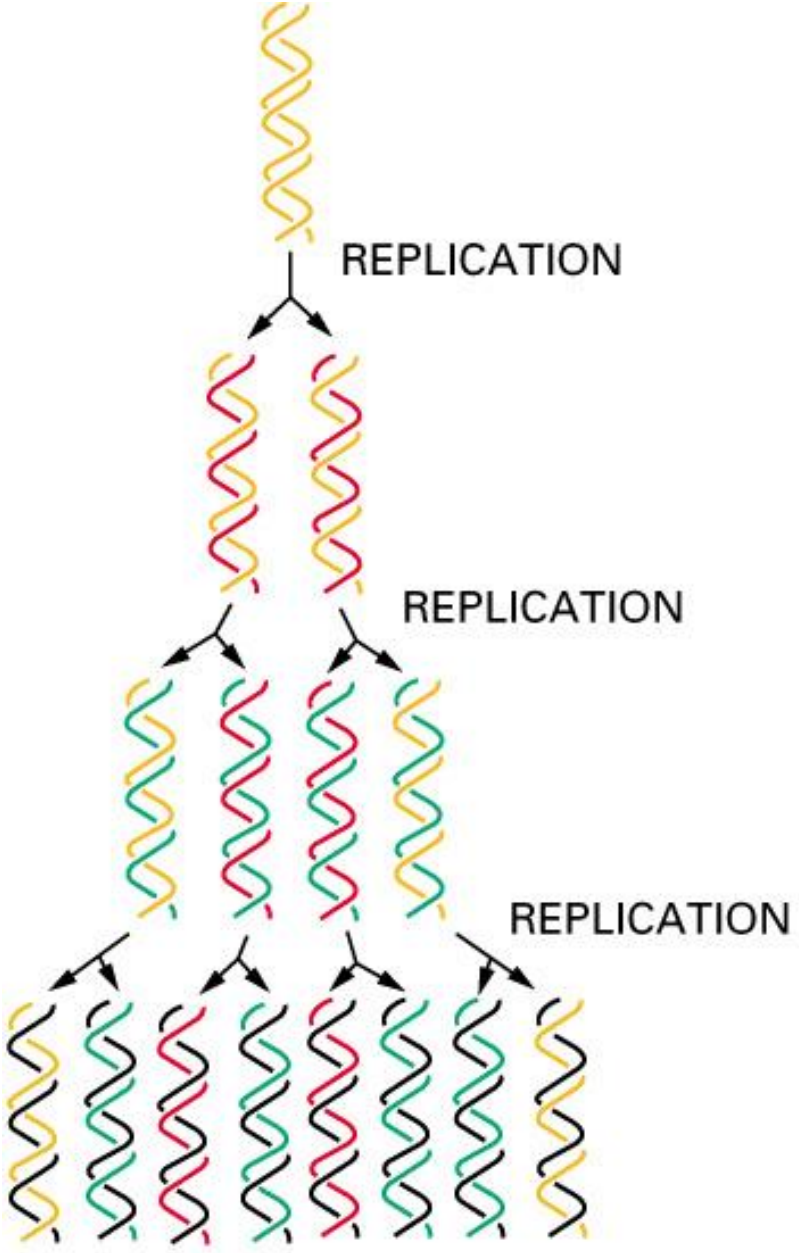


Figure 5–5. Molecular Biology of the Cell, 4th Edition.

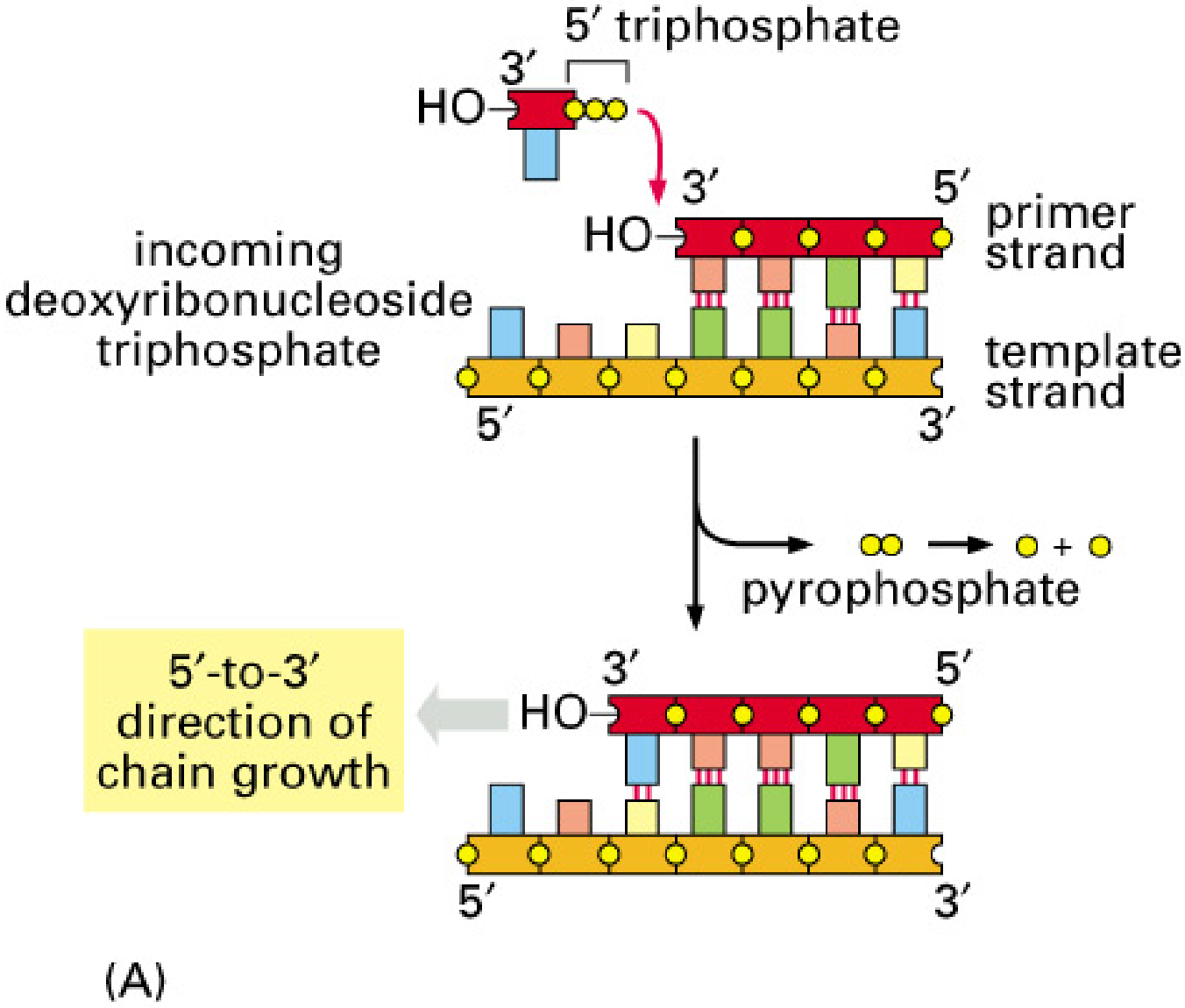


Figure 4–5 part 1 of 2. Molecular Biology of the Cell, 4th Edition.

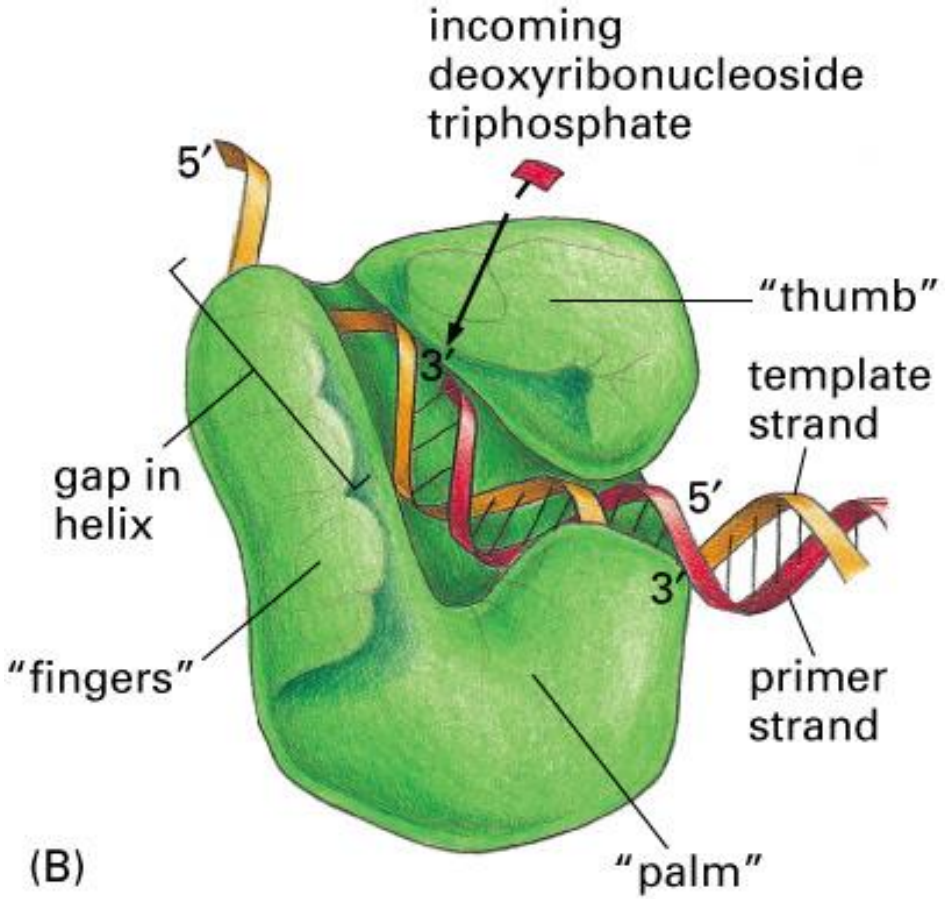
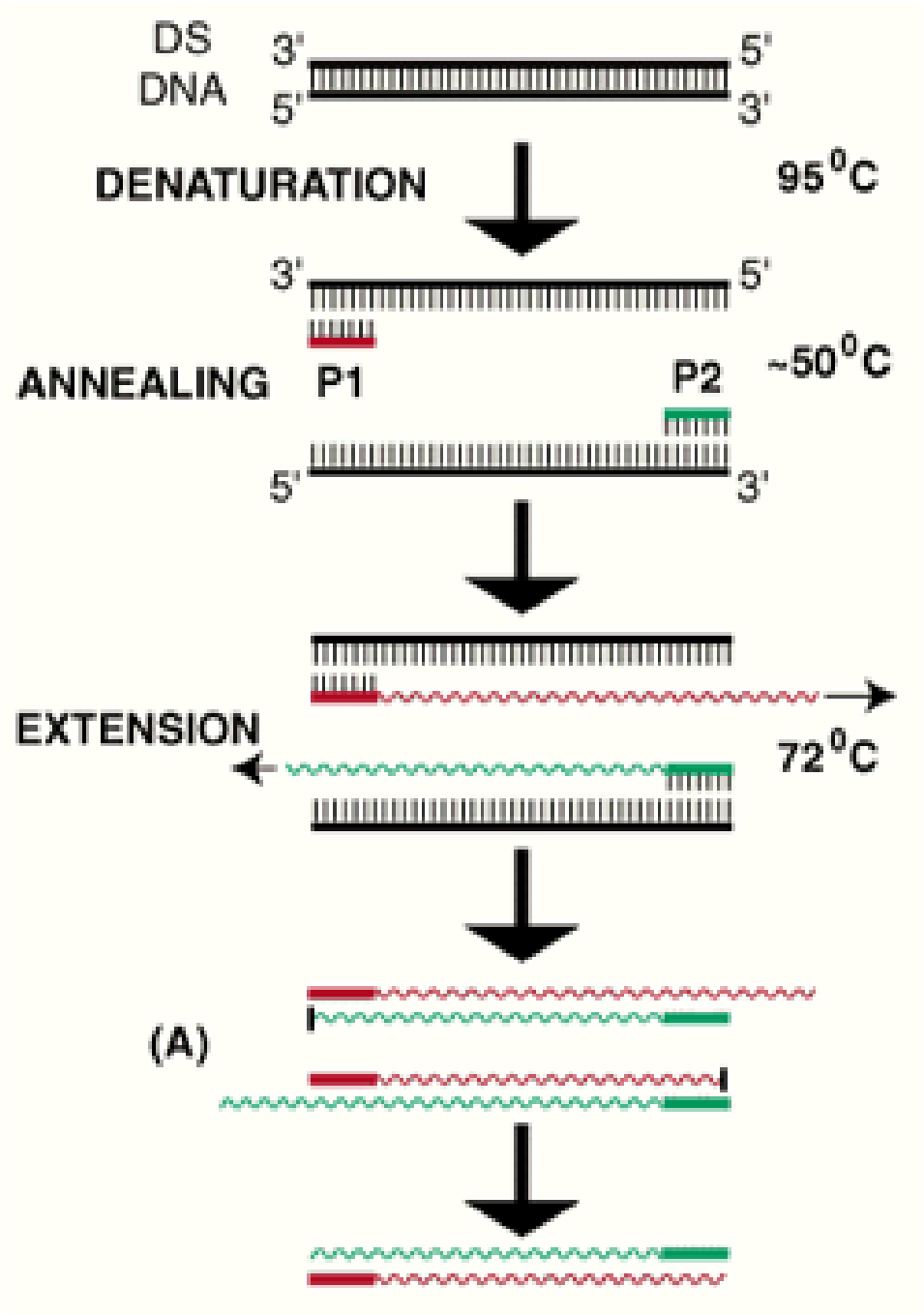
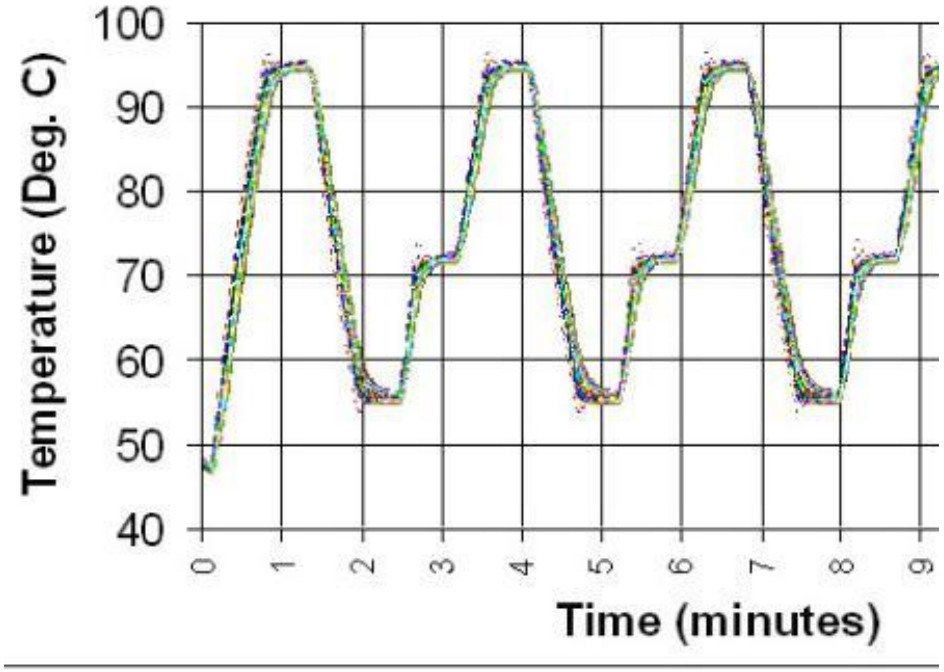


Figure 5–4 part 2 of 2. Molecular Biology of the Cell, 4th Edition.


DS
DNA
3'
5'
5'
3'
DENATURATION
95 0C
3'
5'
ANNEALING
P1
P2
~50 0C
5'
3'
EXTENSION
72 0C
(A)

PCR

PCR machines ($1000-7000)



PCR mutagenesis example 1

The ends of PCR products can be easily manipulated. For example we can add sites for restriction endonucleases, making it easier to clone the products

Target for amplification:

```
5'-GTTTAGAGACCTAGACTA...........ATATTACGCGAGTAGCT-3'
   :::::::::::::::::             :::::::::::::::::
3'-CAAATCTCTGGATCTGAT...........TATAATGCGCTCATCGA-5'
```

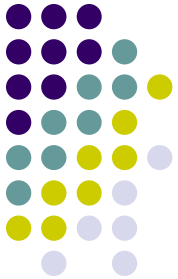
Primers are designed with extra sequences at their 5' ends

```
5'-GTTTAGAGACCTAGACTA...........ATATTACGCGAGTAGCT-3'
                                  ::::::::::::
                              3'-TATAATGCGCTTAAG-5'
      5'-GGATCCTAGACTA-3'
         :::::::::
3'-CAAATCTCTGGATCTGAT...........TATAATGCGCTCATCGA-5'
```

The PCR product now has sites for BamHI and EcoRI at its ends

```
5'-GGATCCTAGACTA...........ATATTACGCGAATTC-3'
   ::::::::::::             ::::::::::::::
3'-CCTAGGATCTGAT...........TATAATGCGCTTAAG-5'
```

PCR mutagenesis example 2



Specific mutations can also be introduced into the middle of a DNA molecule during PCR. This is one strategy among literally hundreds that have been devised for site-directed mutagenesis.

A target sequence:

```
5'-GTTTAGAGACCTAGACTAATATTACGCGAGTAGCT.....-3'
   :::::::::::::::::::::::::::::::::::
3'-CAAATCTCTGGATCTGATTATAATGCGCTCATCGA.....-5'
```

Primers are designed that pair properly at the ends, but mutations are added in the middle (the second primer at the right end of the product is not shown)

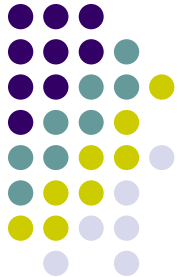
```
          5'-GACCTAGACGCCTATTACGCGA-3'
            :::::::::   ::::::::::
3'-CAAATCTCTGGATCTGATTATAATGCGCTCATCGA.....-5'
```

The PCR product contains the mutation

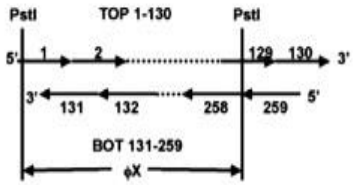
```
 5-GACCTAGACGCCTATTACGCGAGTAGCT.....-3'
   ::::::::::::::::::::::::::::::
3'-CTGGATCTGCGGATAATGCGCTCATCGA.....-5'
```

Stop to Ala

Building a synthetic genome via PCA (polymerase cycle assembly)



- http://www.venterinstitute.org/research/#syntheticbiology
- Smith, H. O., C. A. Hutchison, 3rd, et al. (2003). "Generating a synthetic genome by whole genome assembly: phiX174 bacteriophage from synthetic oligonucleotides." Proc Natl Acad Sci U S A **100**(26): 15440-5.
- http://www.ncbi.nlm.nih.gov/pubmed/18218864?dopt=Citation (Complete chemical synthesis, assembly, and cloning of a Mycoplasma genitalium genome)
- Use small oligonucleotides to assemble larger and larger pieces of DNA using a PCR methodology called PCA (polymerase cycle assembly)

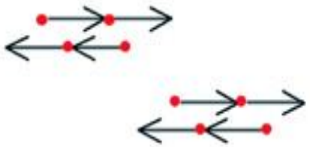


Design oligos — 8 hrs

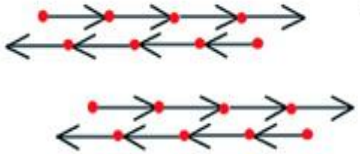
Synthesize oligos — ~4 days

Purify top strand and bottom strand oligos in separate pools by gel electrophoresis — 2 days

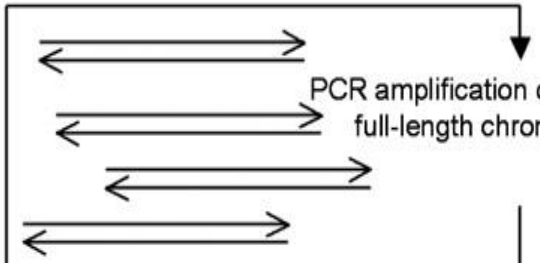
5'-phosphorylate the oligos — 4 hrs



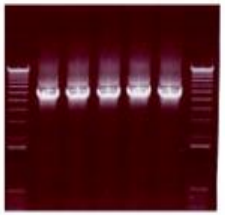
Taq ligate the pooled top and bottom oligos overnight at 55°C — 18 hrs



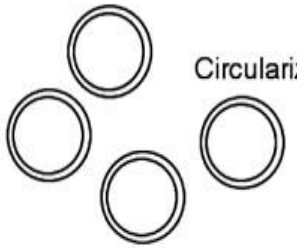
Polymerase cycling assembly (PCA) of ligation products into full-length chromosomes, 35 to 70 cycles — 12 to 24 hrs



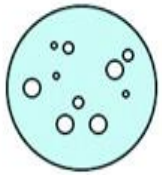
PCR amplification of assembled full-length chromosomes — 3 hrs



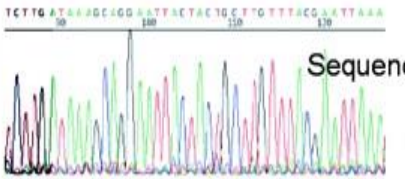
Gel-purify amplified chromosomes — 8 hrs



Circularize the synthetic linear chromosomal DNA so that it is infectious — 4 hrs



Electroporate into *E. coli* and plate for phage plaques — 24 hrs

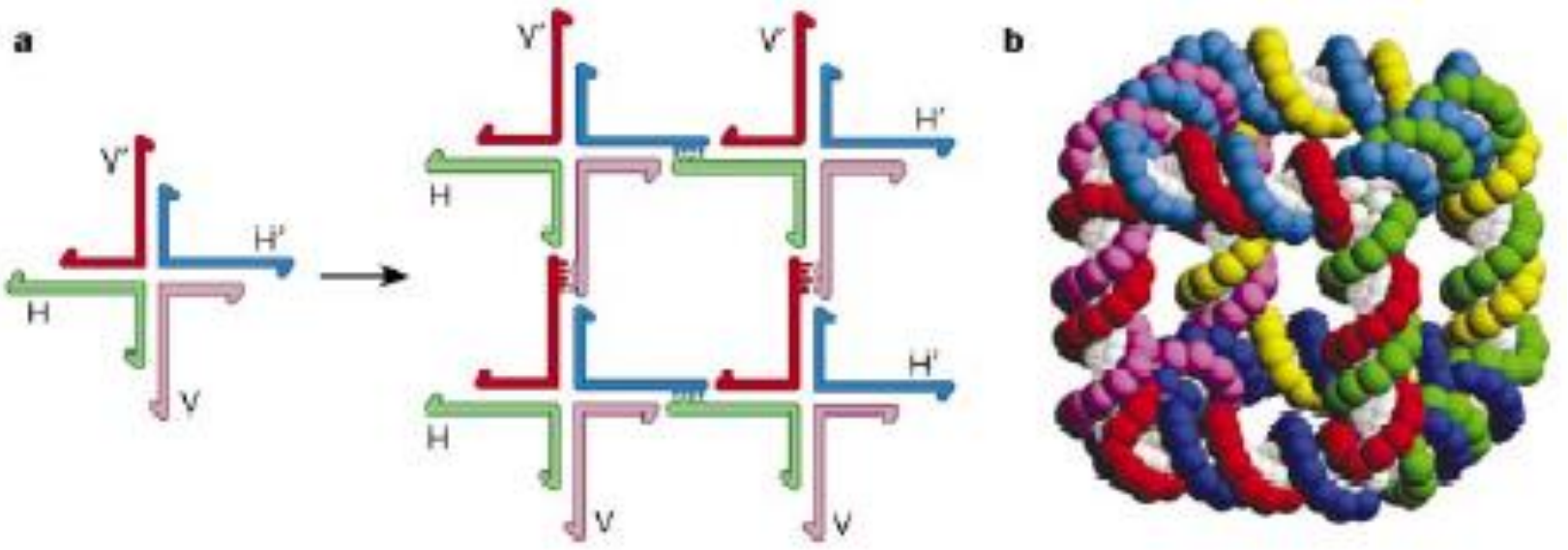


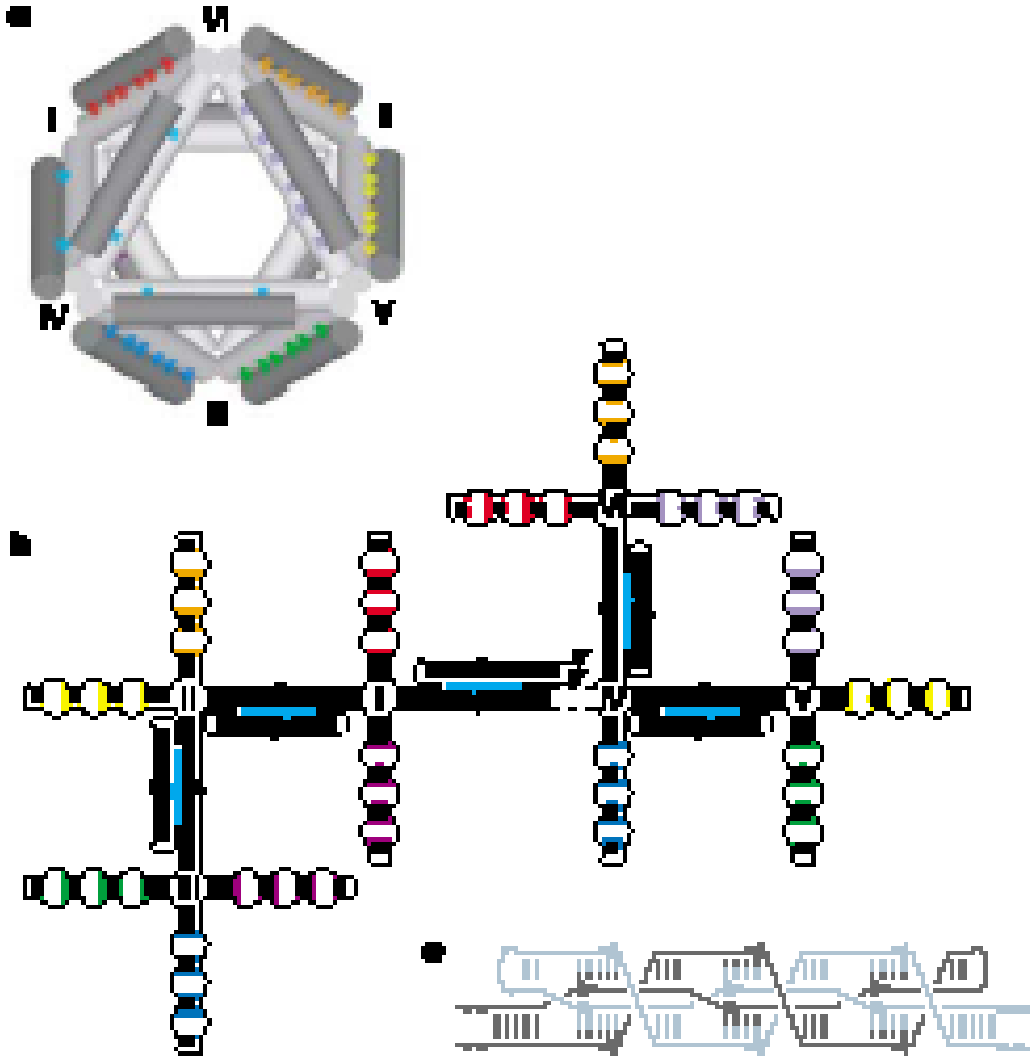
Sequence phage from individual plaques

Total time ~2 weeks

DNA as building blocks

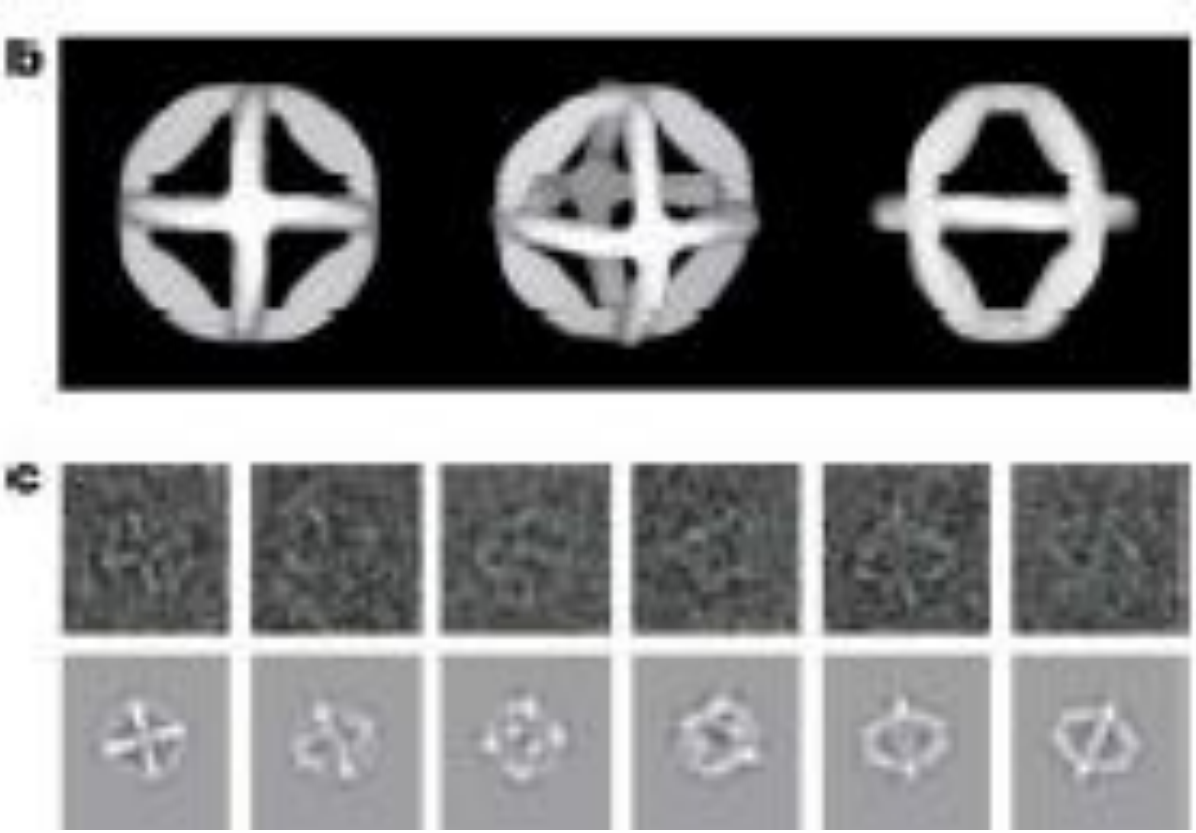
- Using a variety of complementary DNA molecules to build ordered structures
- Cubes, octahedron, stick figures, etc
- Have not been able to clone and replicate





Shih, W. M., Quispe, J. D. & Joyce, G. F. A 1.7-kilobase single-stranded DNA that folds into a nanoscale octahedron. *Nature* 427, 618-21 (2004).{Shih, 2004 #849}

Structurally intact (EM)



Catalytic Antibodies

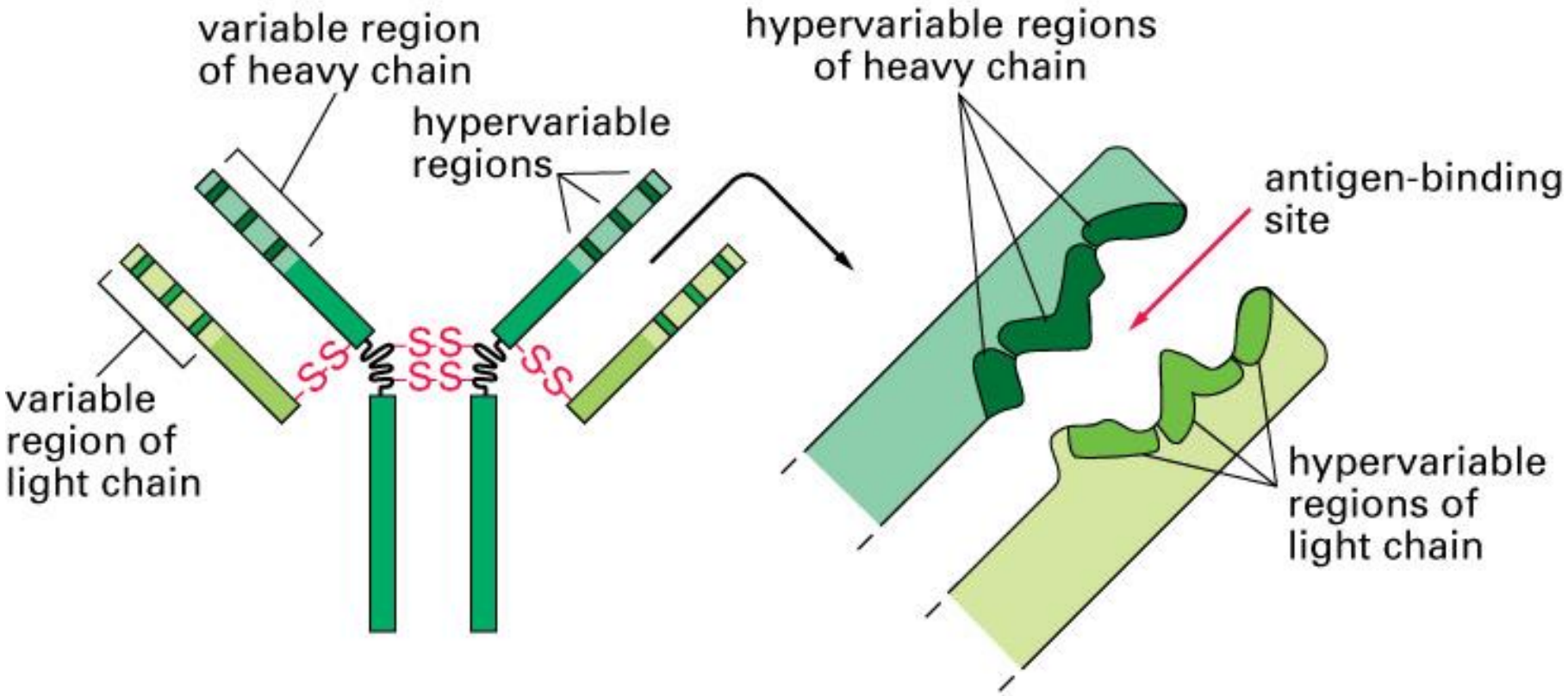


Figure 24–31. Molecular Biology of the Cell, 4th Edition.

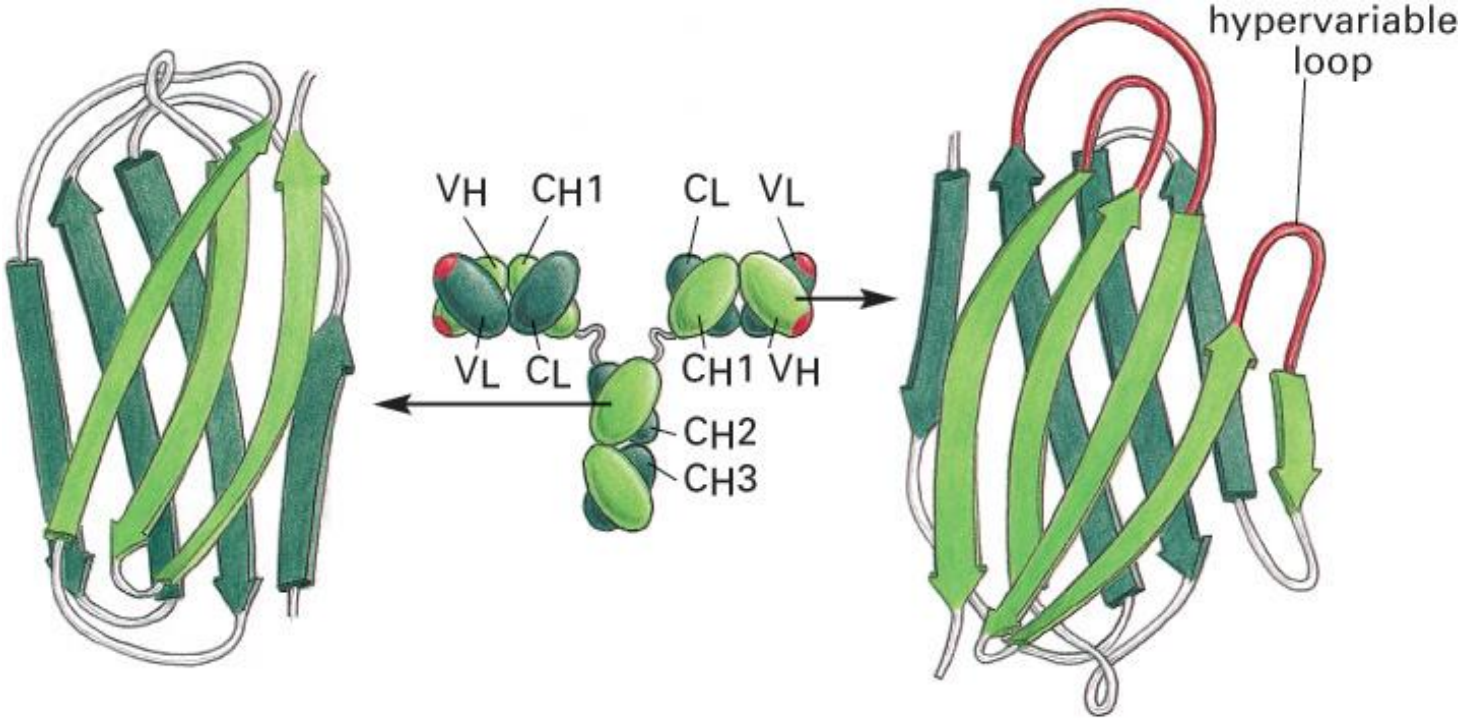
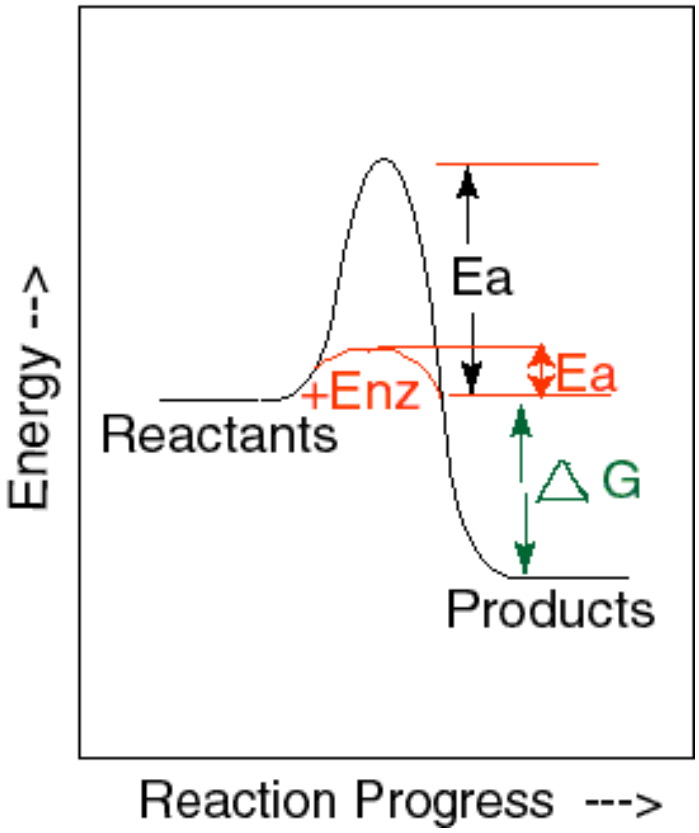
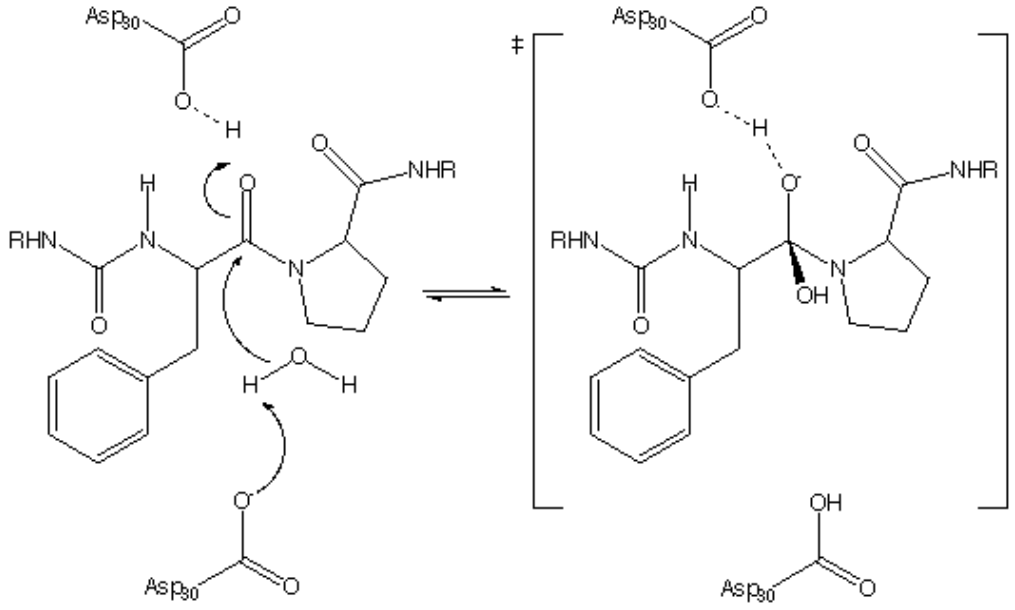
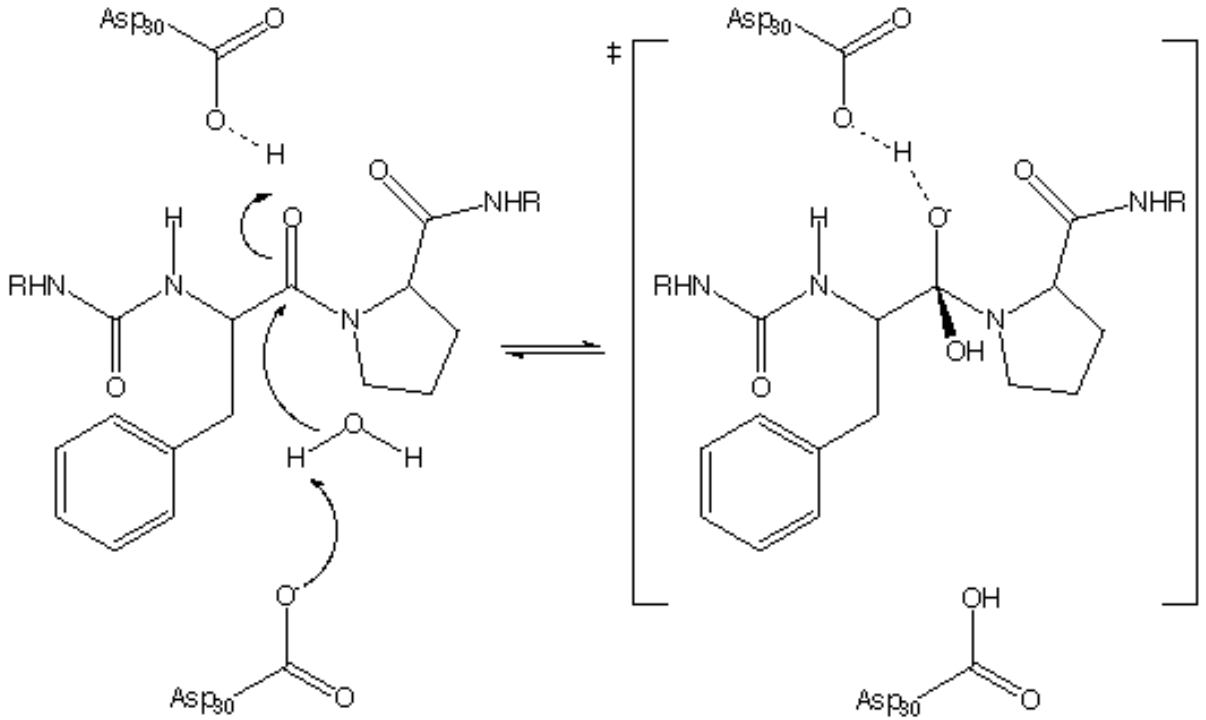


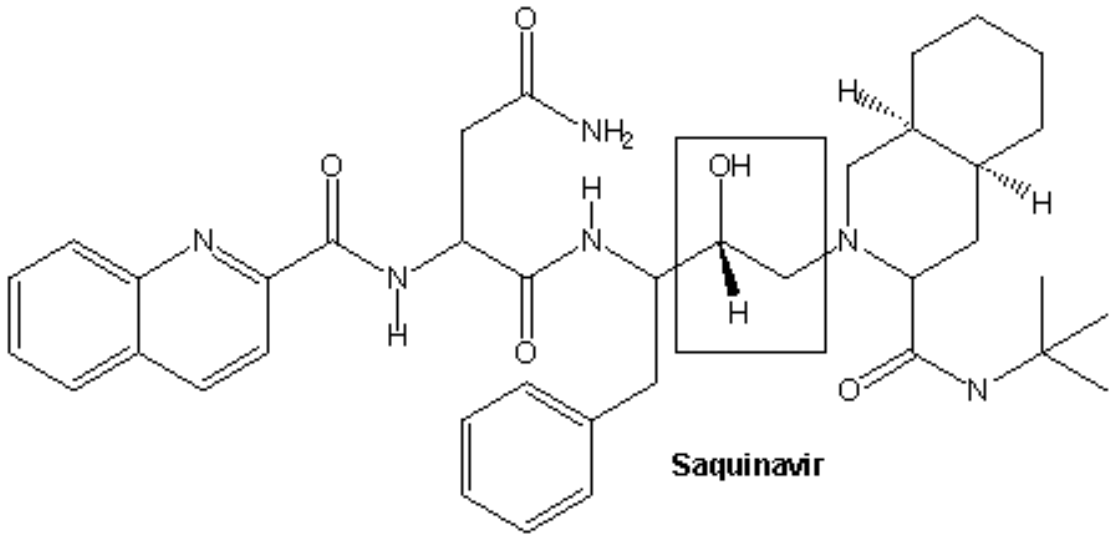
Figure 24–34. Molecular Biology of the Cell, 4th Edition.

Remember transition state theory









Saquinavir

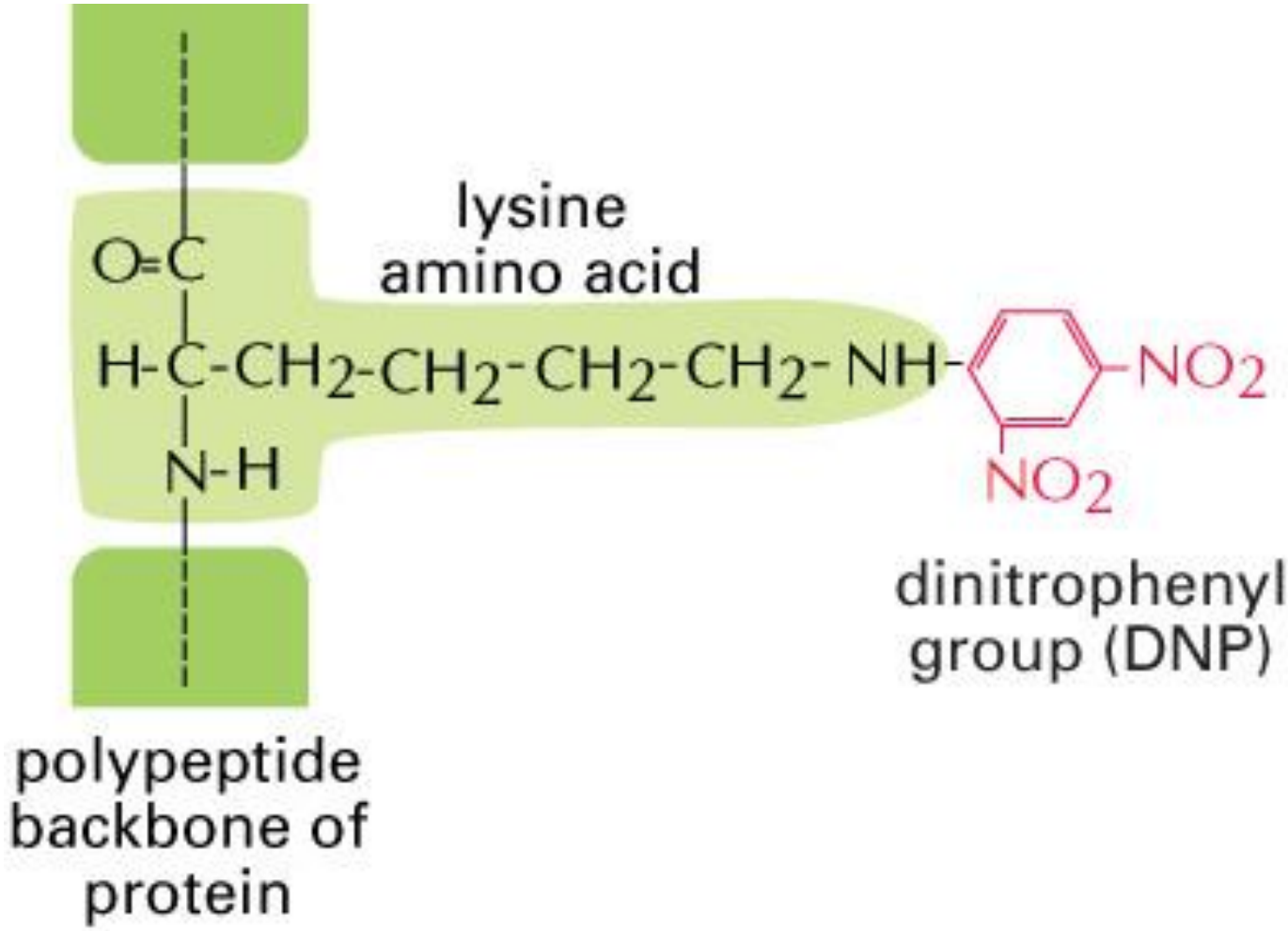
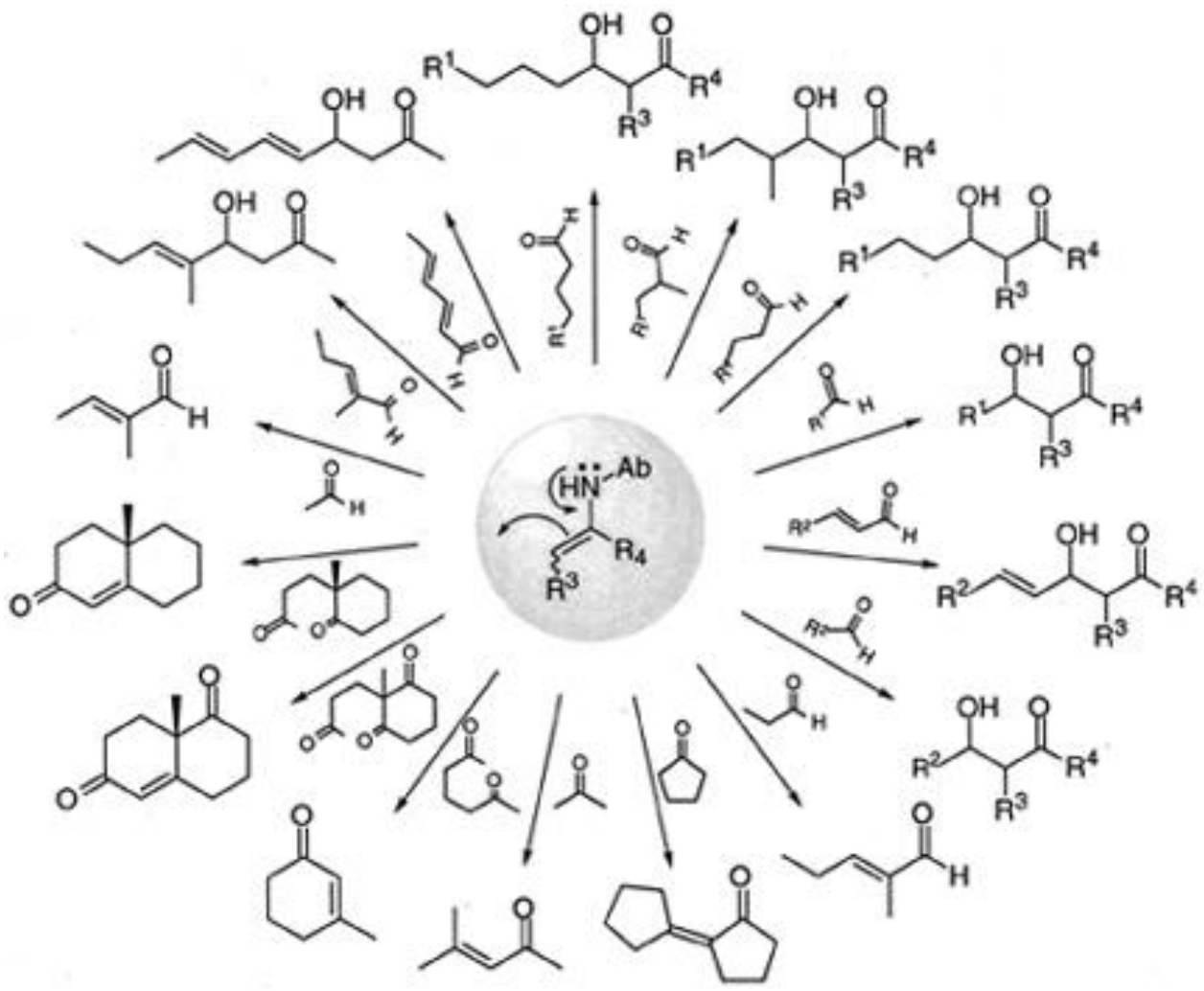


Figure 24–9. Molecular Biology of the Cell, 4th Edition.

Commercially available

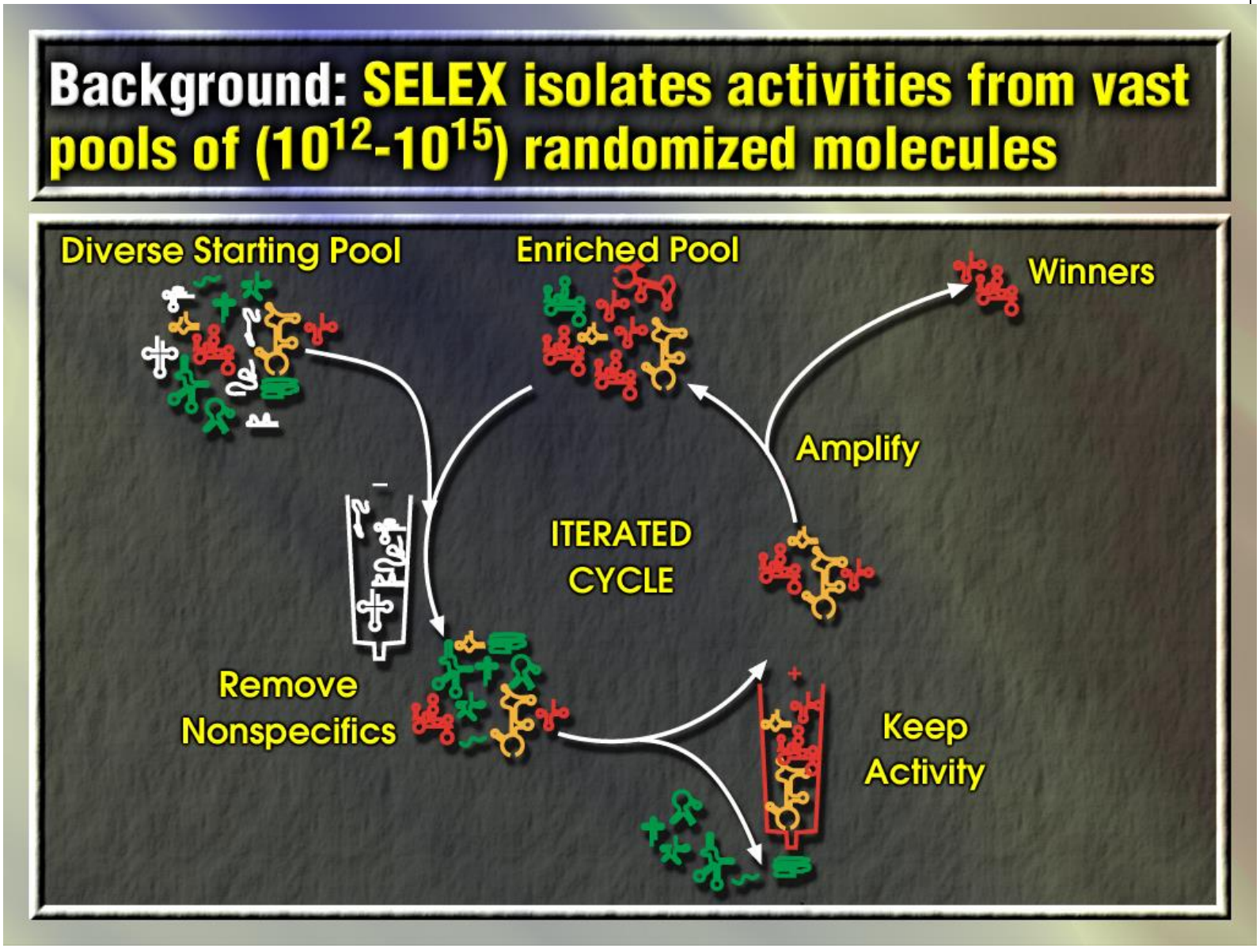


Catalytic antibodies summary

- Suitable for a wide variety of applications
- Transition state analogue required
- Catalytic efficiencies far below “real” enzymes but very good nonetheless

SELEX for RNA Aptamers

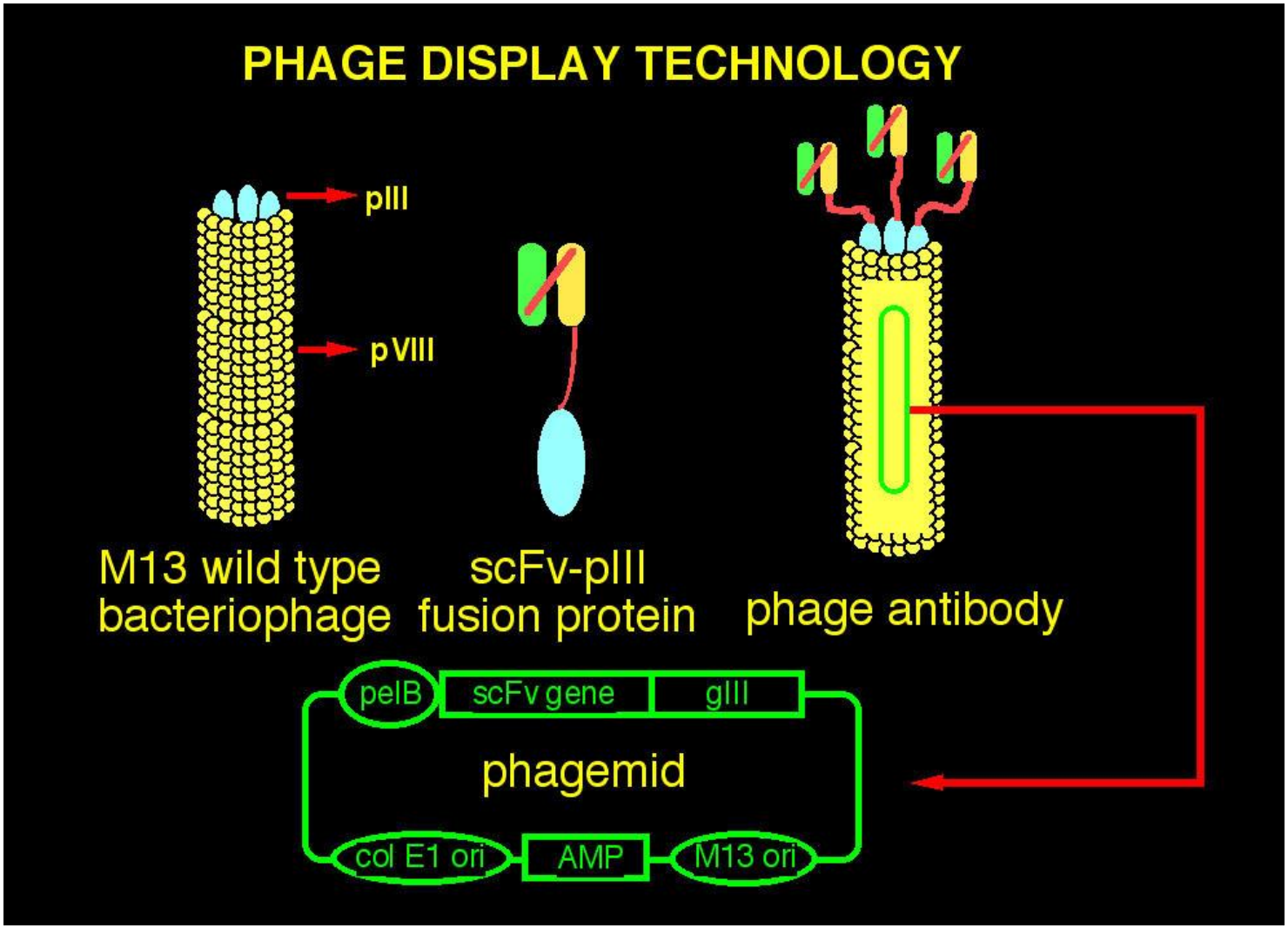
systematic evolution of ligands by exponential enrichment

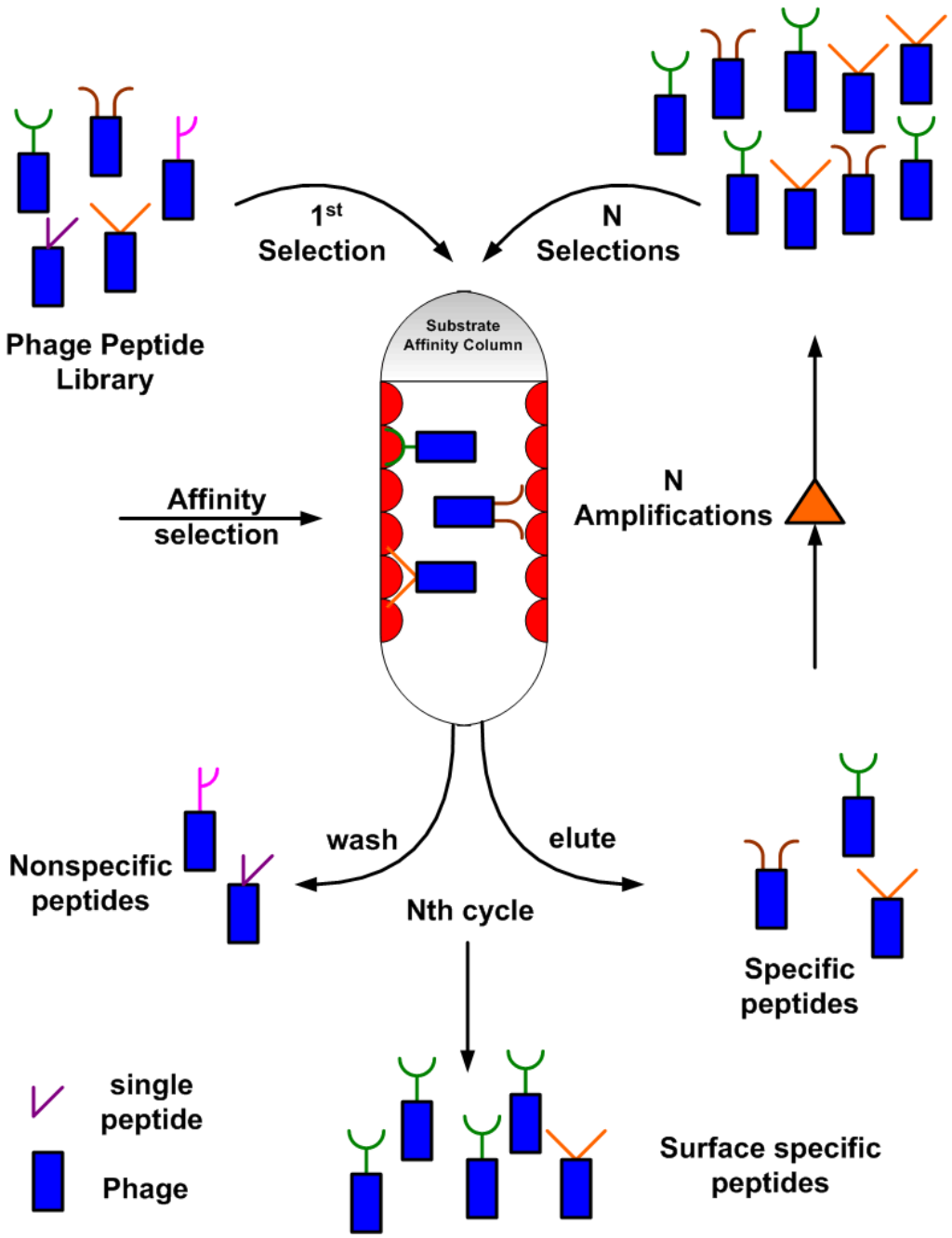


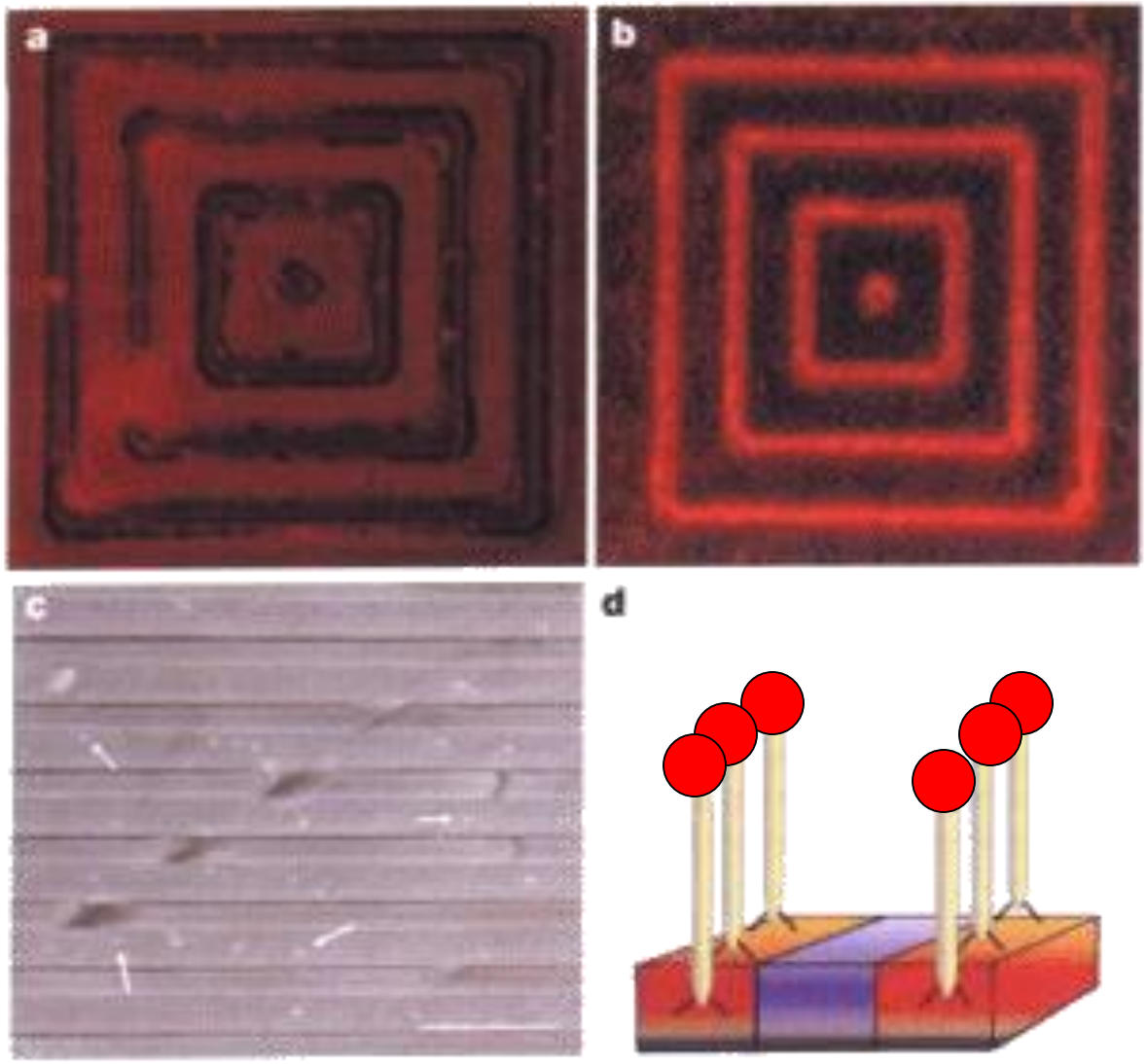
SELEX basics

- Random RNA (or DNA) pool (10^{12} -10^{15})
  - From DNA synthesizer
- Any stable substance suitable for selection
  - proteins, transition states, inorganic, etc
- Rapid optimization
- Typically 20-30 bases
- Variety of applications
- Immobilization can be problematic
- http://aptamer.icmb.utexas.edu/

Phage display of peptides




1st Selection
N Selections
Phage Peptide Library
Substrate Affinity Column
Affinity selection
N Amplifications
wash
elute
Nonspecific peptides
Nth cycle
Specific peptides
single peptide
Phage
Surface specific peptides



GaAs

(*Whaley et al. (2000) Nature 405, 665-668*).

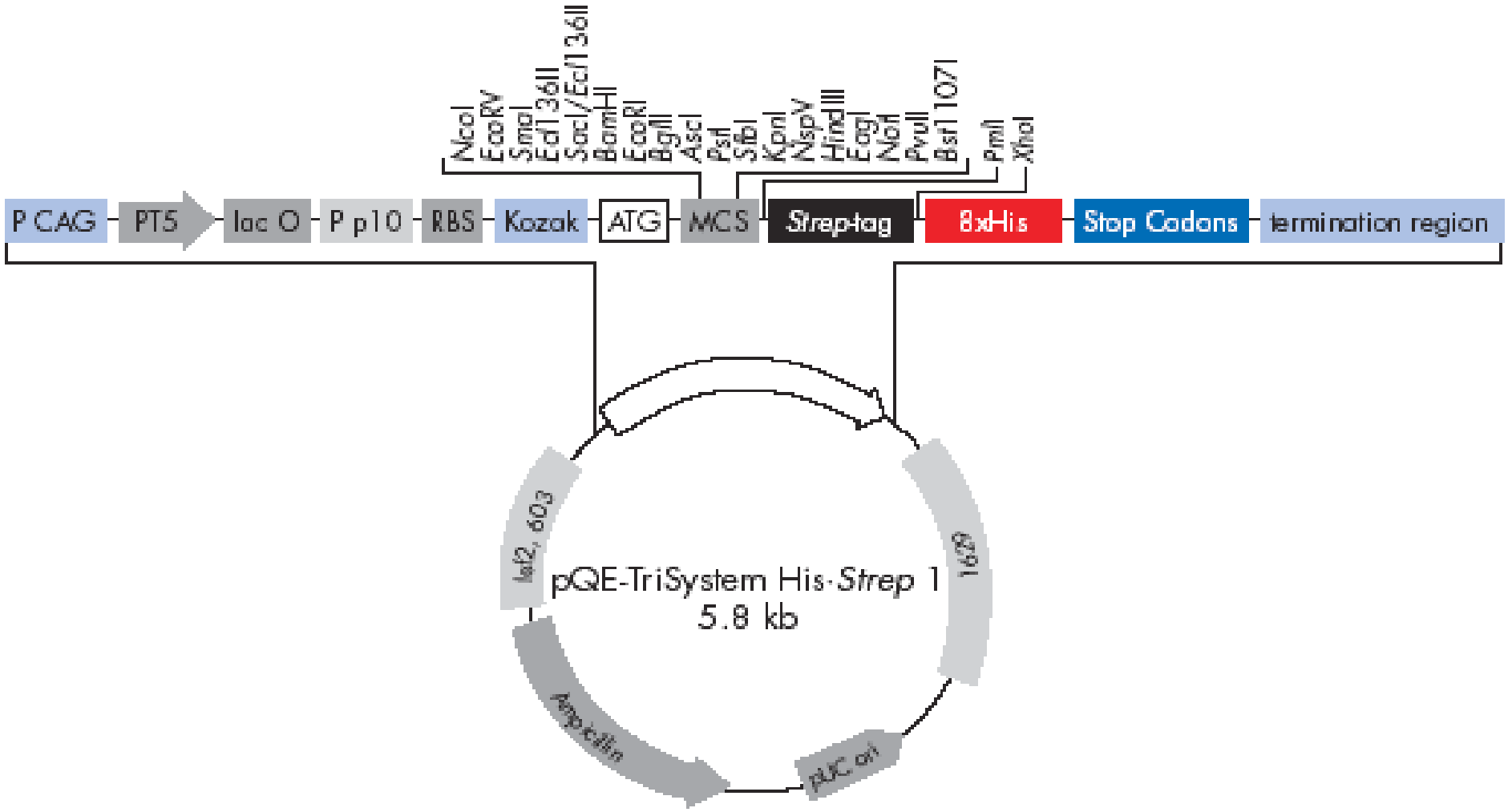
Workshop

- Are other molecules beside DNA/RNA and proteins capable of a SELEX type procedure?
- During such a selection process what else is being selected for beside the target?

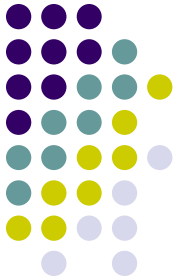
Tagged proteins

- Using expression systems
  - Introduce a variety of tags
    - Poly-His Tag, Biotin Tag, Flag Tag, etc
    - Have specific affinities
- Use tagged proteins as building blocks
  - Tracks of specific proteins
  - Localization of motors
  - etc

Remember expression vector



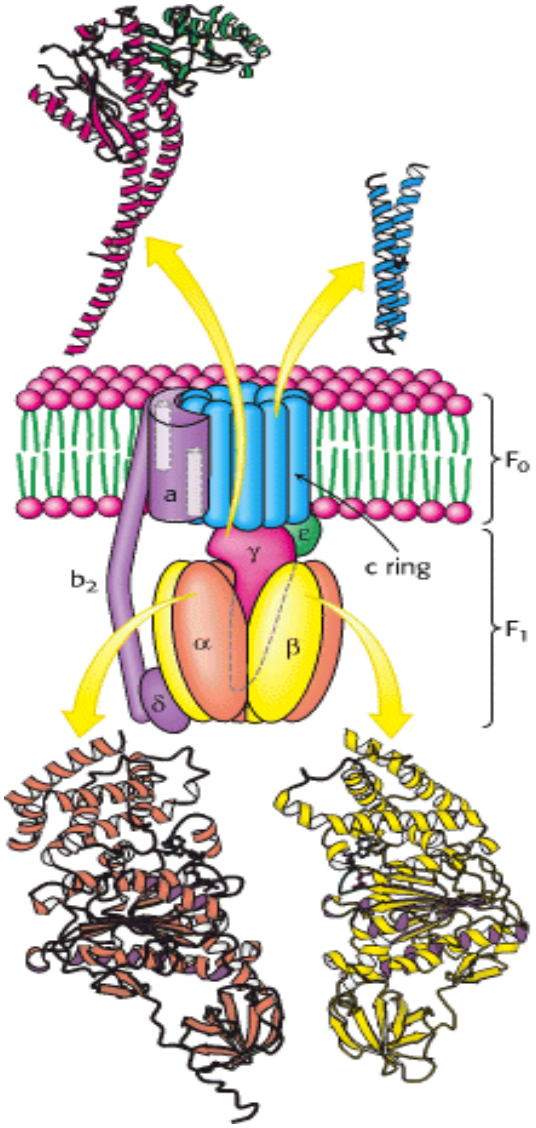
Detailed example 1: Patterned ATP synthase motors

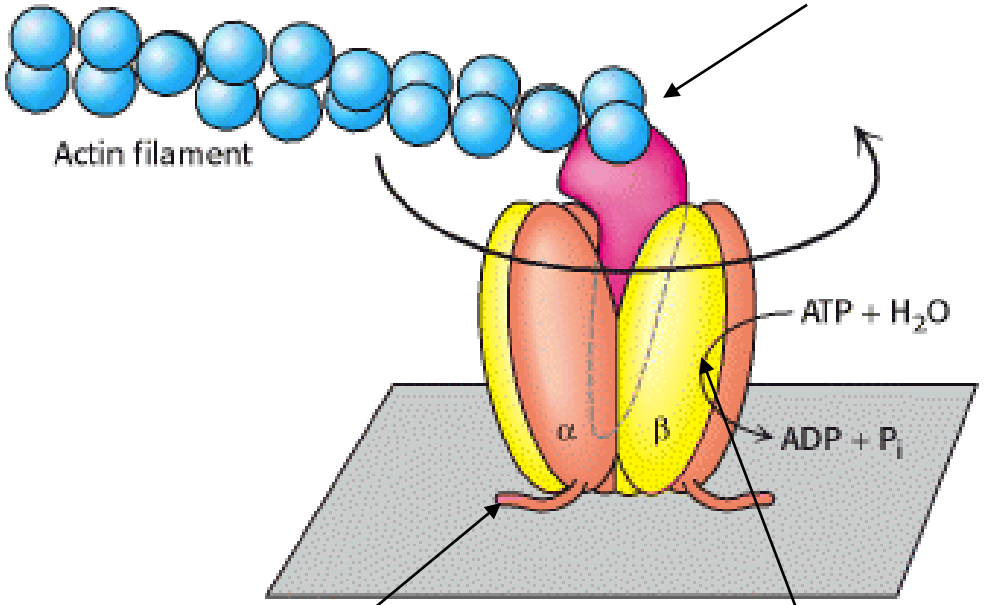


- Goals
  - Attach ATP synthase rotary motor in defined positions on a substrate.
  - Attach metallic rods to ATP synthase
  - Drive rotary motion with ATP
  - Control specific motors through mutation

Soong et al Science, 2000, Vol 290, pg 1555
Lui et al Nature Materials,2002, Vol 1, pg 173

Remember ATPsynthase



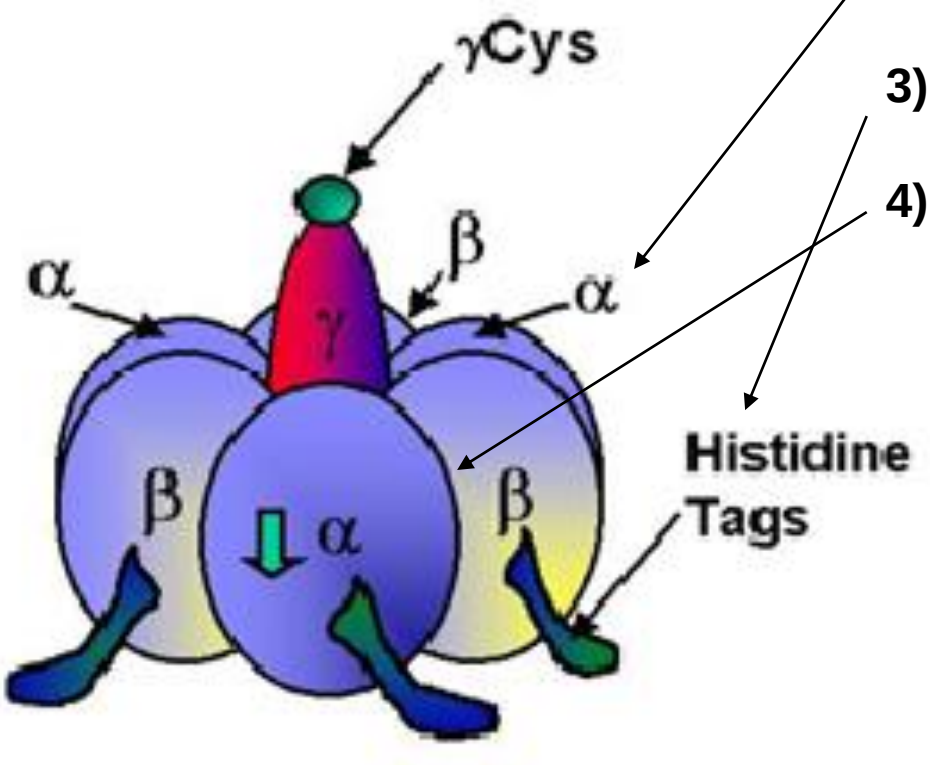


And here for TAG

Make mutations here for TAG

And here for control

Step 1: Design and Create Tags



1) Mutate γSer^{107} to Cys
   To attach biotin and propellers
2) Mutate αCys^{193} to Ser
   To remove nonspecific biotin label
3) Introduce His_{10} tag to beta subunit
   To attach nickel surface
4) Mutate Active Site
   To control specific propellers

Remember PCR

The ends of PCR products can be easily manipulated. For example we can add sites for restriction endonucleases, making it easier to clone the products

Target for amplification:

```
5'-GTTTAGAGACCTAGACTA...........ATATTACGCGAGTAGCT-3'
   :::::::::::::::::::           :::::::::::::::::
3'-CAAATCTCTGGATCTGAT...........TATAATGCGCTCATCGA-5'
```

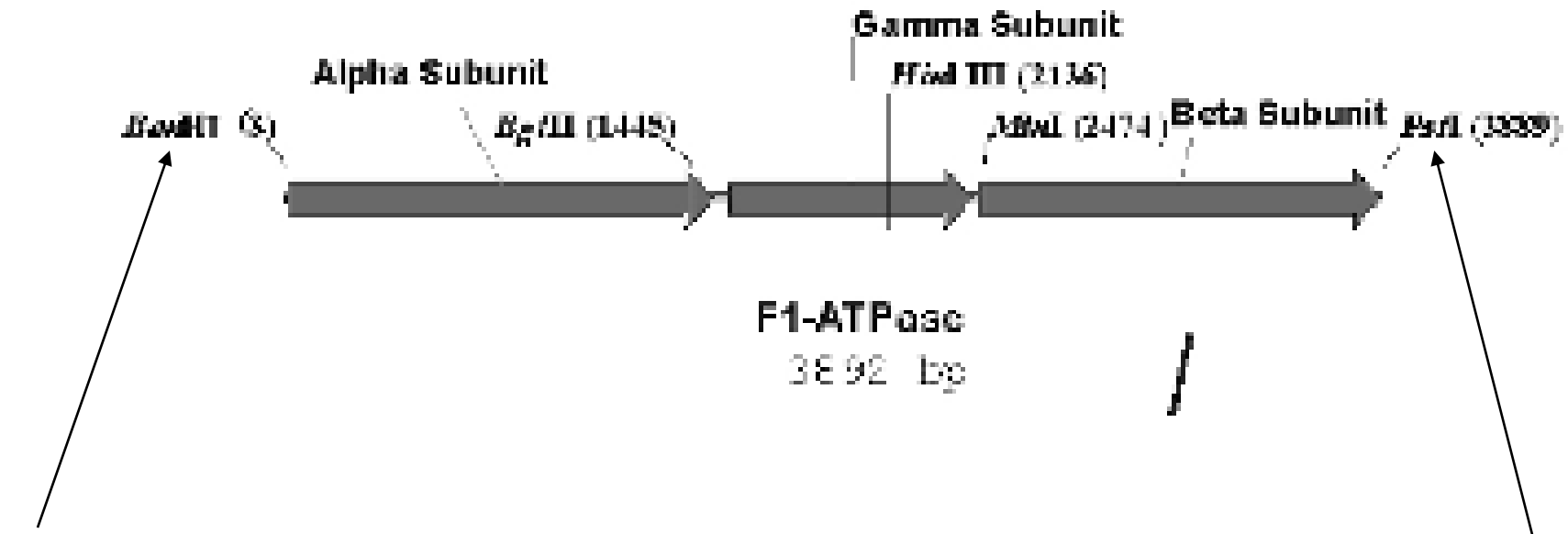
Primers are designed with extra sequences at their 5' ends

```
5'-GTTTAGAGACCTAGACTA...........ATATTACGCGAGTAGCT-3'
                                   :::::::::::
                                3'-TATAATGCGCTTAAG-5'
      5'-GGATCCTAGACTA-3'
         :::::::::
3'-CAAATCTCTGGATCTGAT...........TATAATGCGCTCATCGA-5'
```

The PCR product now has sites for BamHI and EcoRI at its ends

```
5'-GGATCCTAGACTA...........ATATTACGCGAATTC-3'
   ::::::::::::           :::::::::::::::
3'-CCTAGGATCTGAT...........TATAATGCGCTTAAG-5'
```

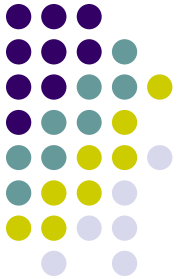
Mutations and cloning



Introduced during PCR

Introduced during PCR

PCR mutagenesis



Specific mutations can also be introduced into the middle of a DNA molecule during PCR. This is one strategy among literally hundreds that have been devised for site-directed mutagenesis.

A target sequence:

```
5'-GTTTAGAGACCTAGACTAATATTACGCGAGTAGCT.....-3'
   :::::::::::::::::::::::::::::::::::::
3'-CAAATCTCTGGATCTGATTATAATGCGCTCATCGA.....-5'
```

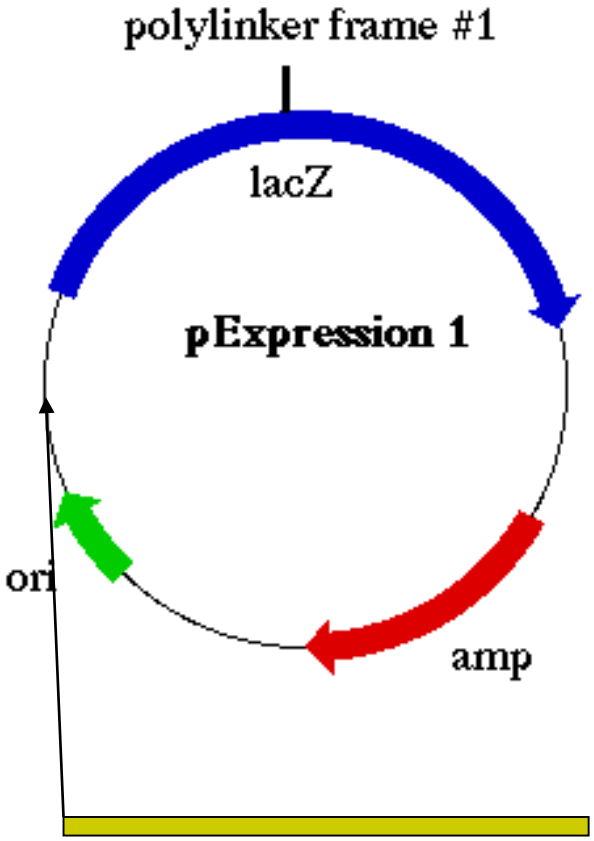
Primers are designed that pair properly at the ends, but mutations are added in the middle (the second primer at the right end of the product is not shown)

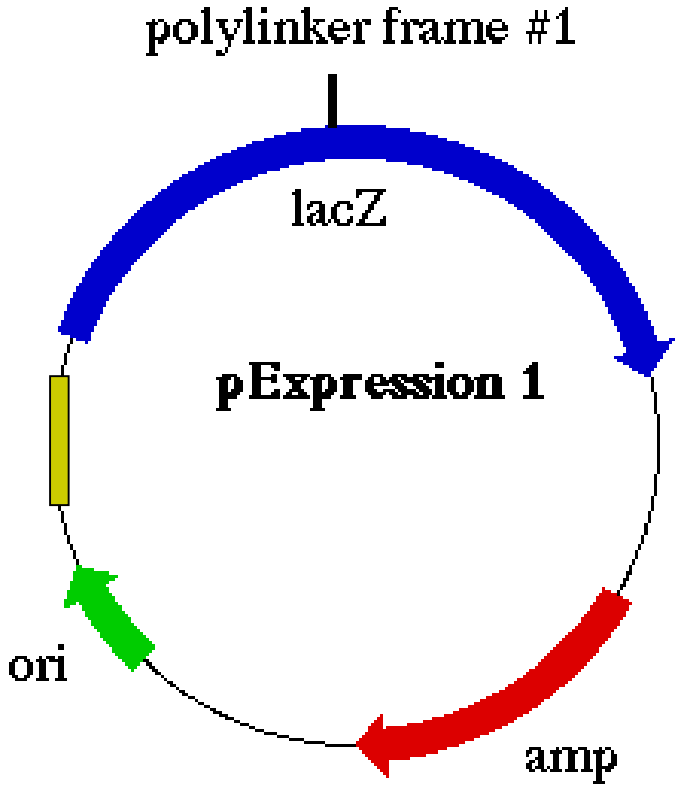
```
          5'-GACCTAGACGCCTATTACGCGA-3'
             ::::::::::    ::::::::::
3'-CAAATCTCTGGATCTGATTATAATGCGCTCATCGA.....-5'
```

The PCR product contains the mutation

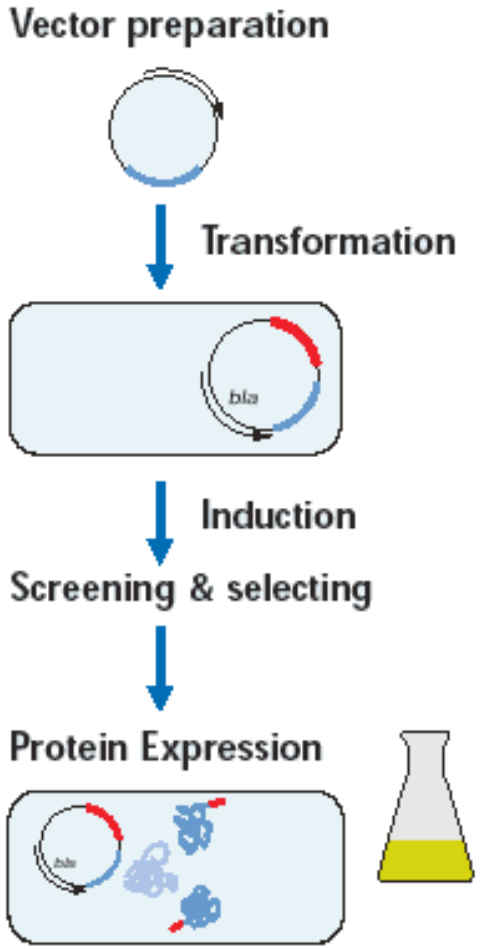
```
          5-GACCTAGACGCCTATTACGCGAGTAGCT.....-3'
             ::::::::::::::::::::::::::::::
          3'-CTGGATCTGCGGATAATGCGCTCATCGA.....-5'
```

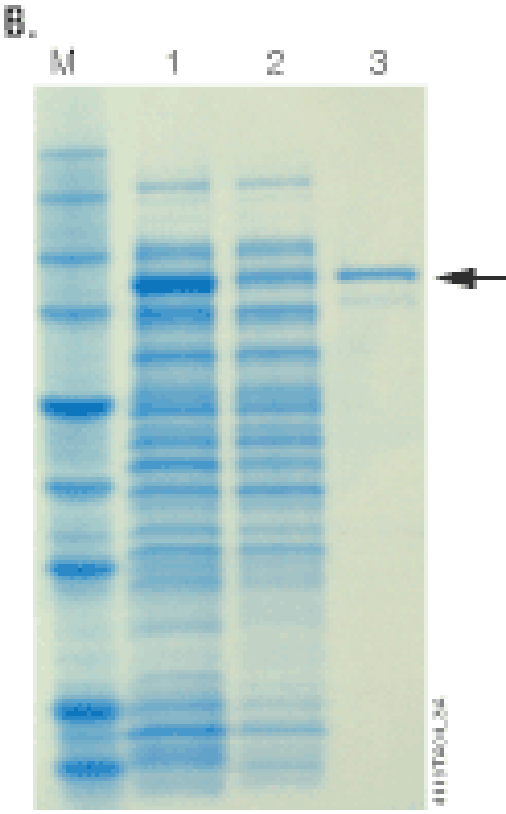
Put mutant genes into expression vector



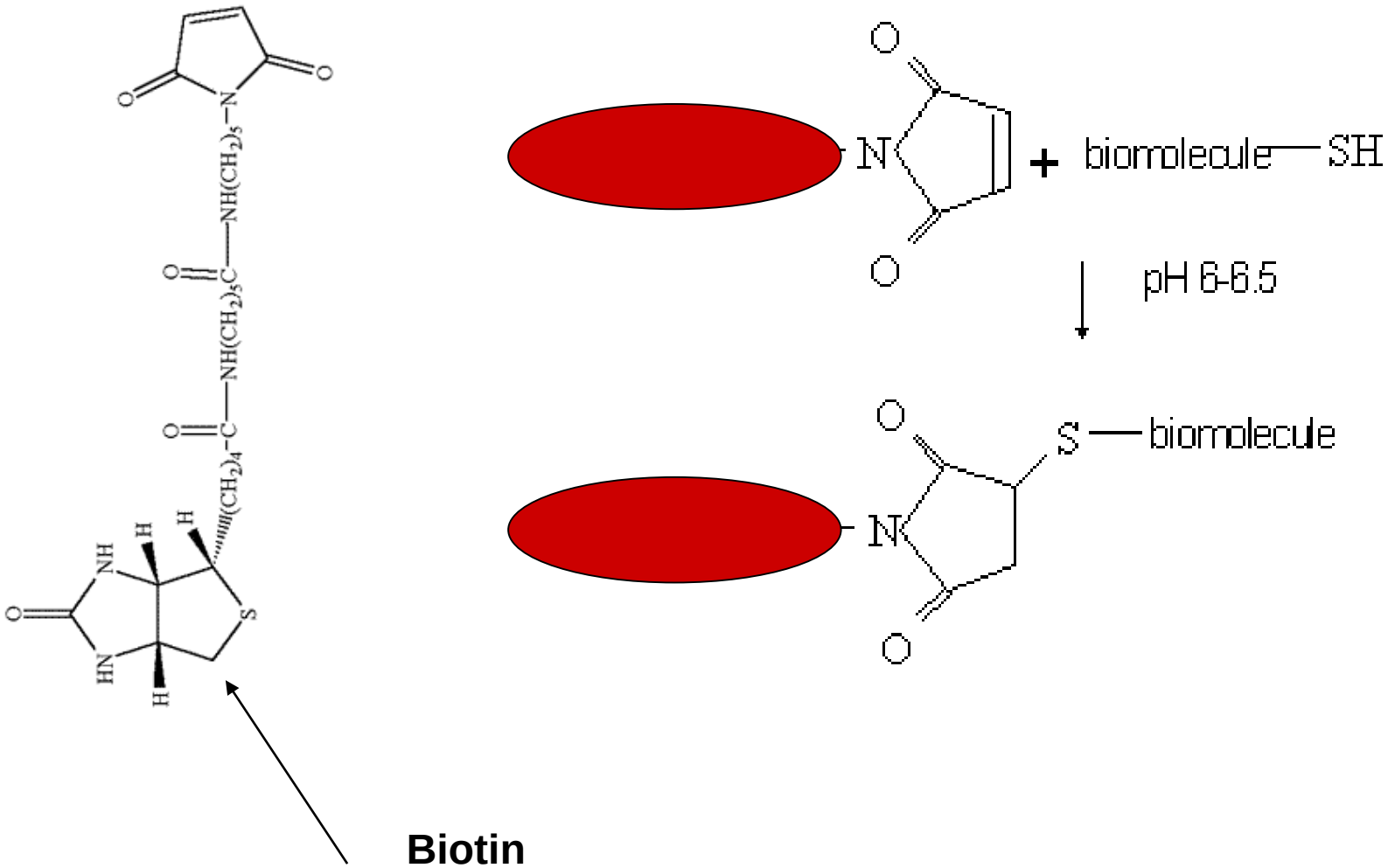


Transfect plasmid into bacteria and express protein



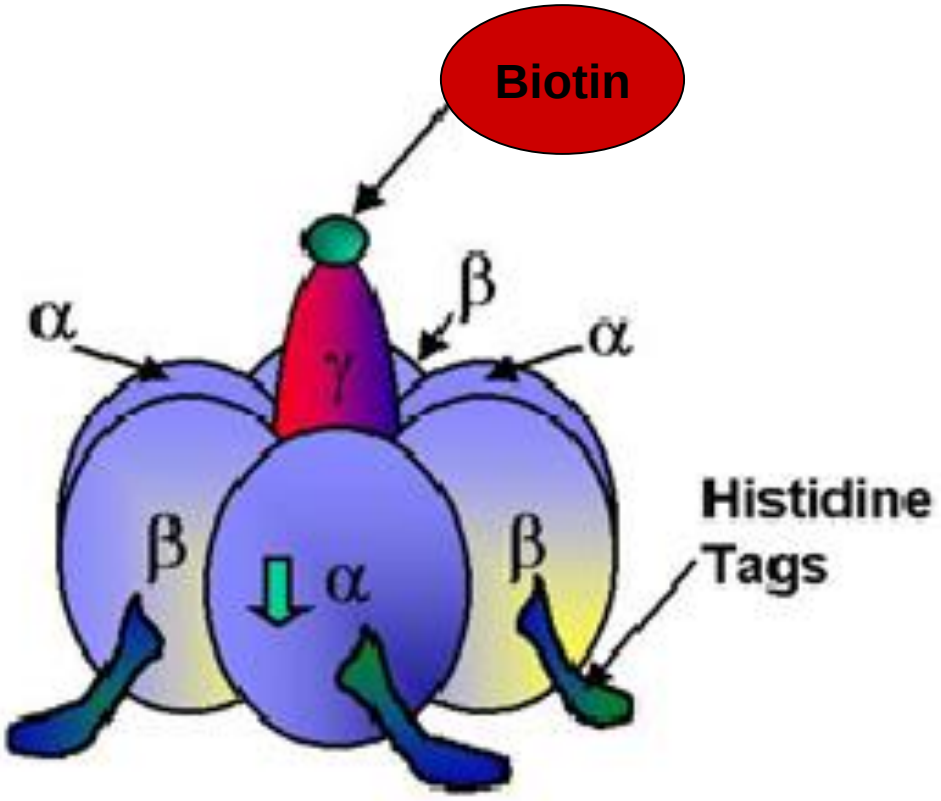


Biotin Labeling

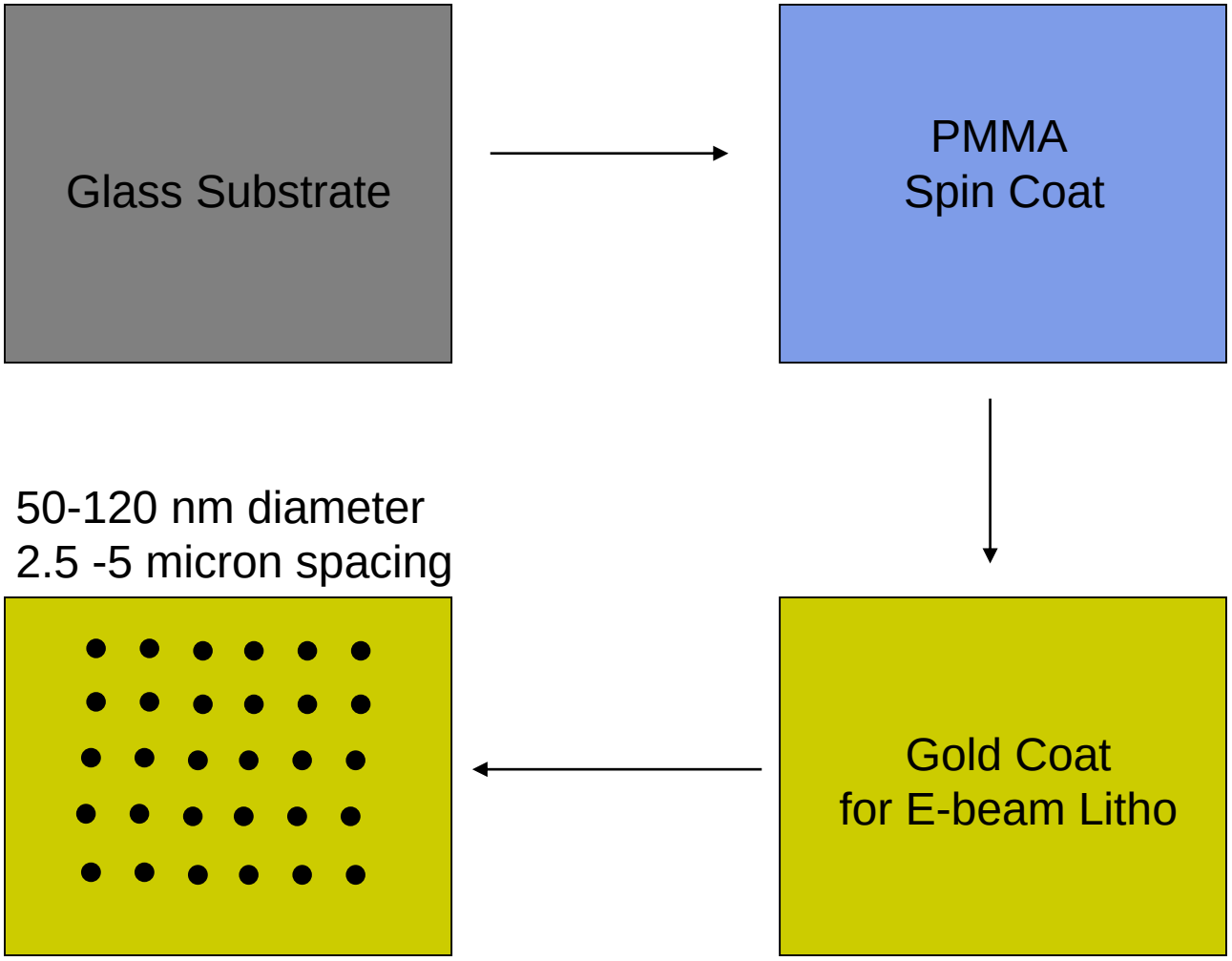


Biotin

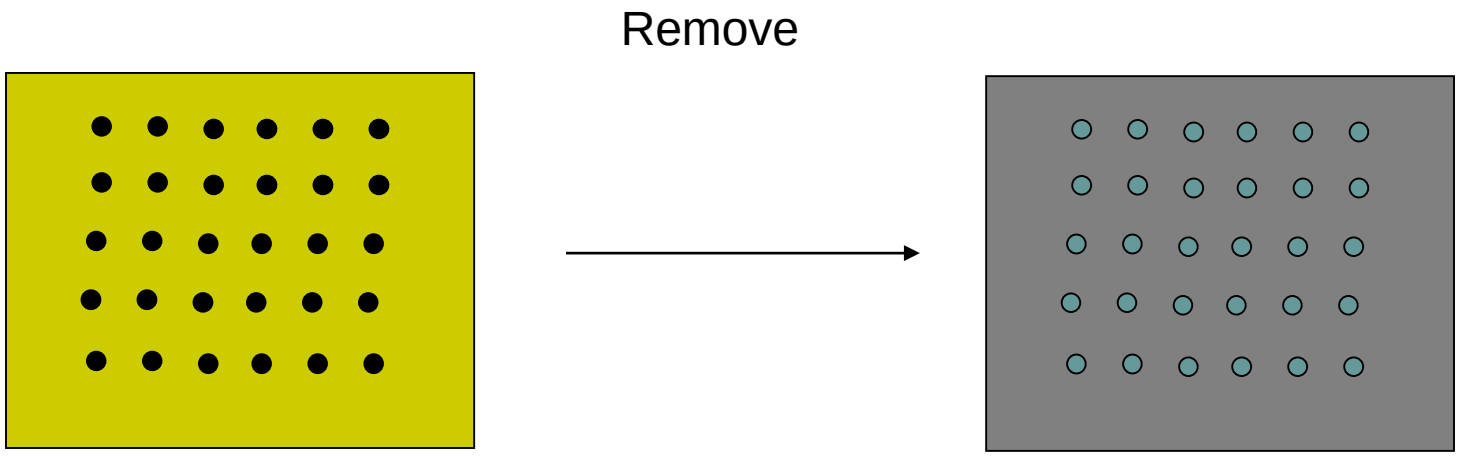
So now we have the following



Step 2: Design and Create patterned surface

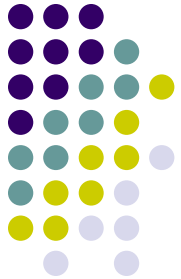


Develop and Ni coat pillar



200 nm high nickel capped pillars

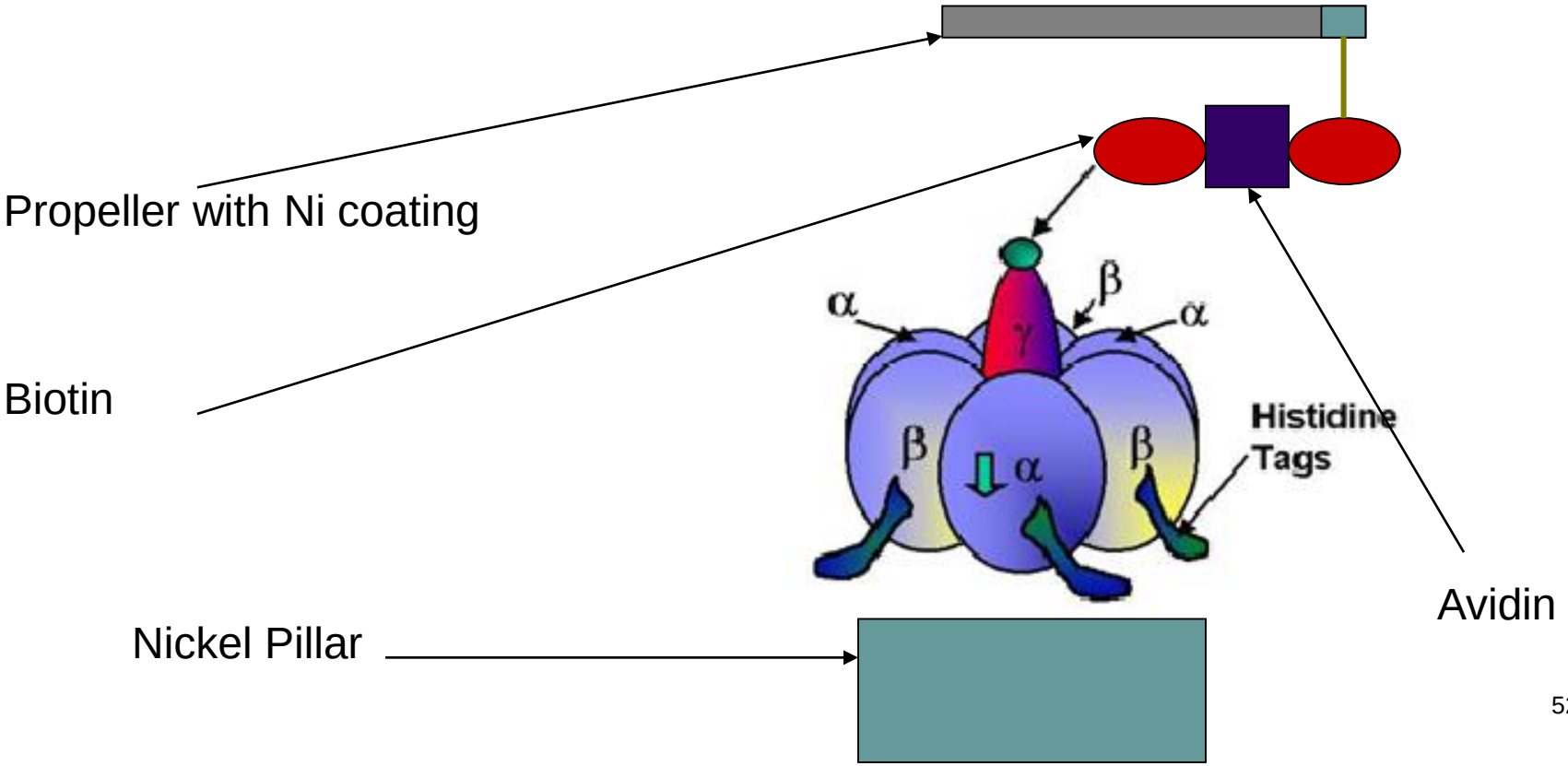
Similar strategy to produce propellers



- 750-150 nm propellers
- Electron gun evaporation of Ni
- His10-Cys peptide
- Biotin-label as above

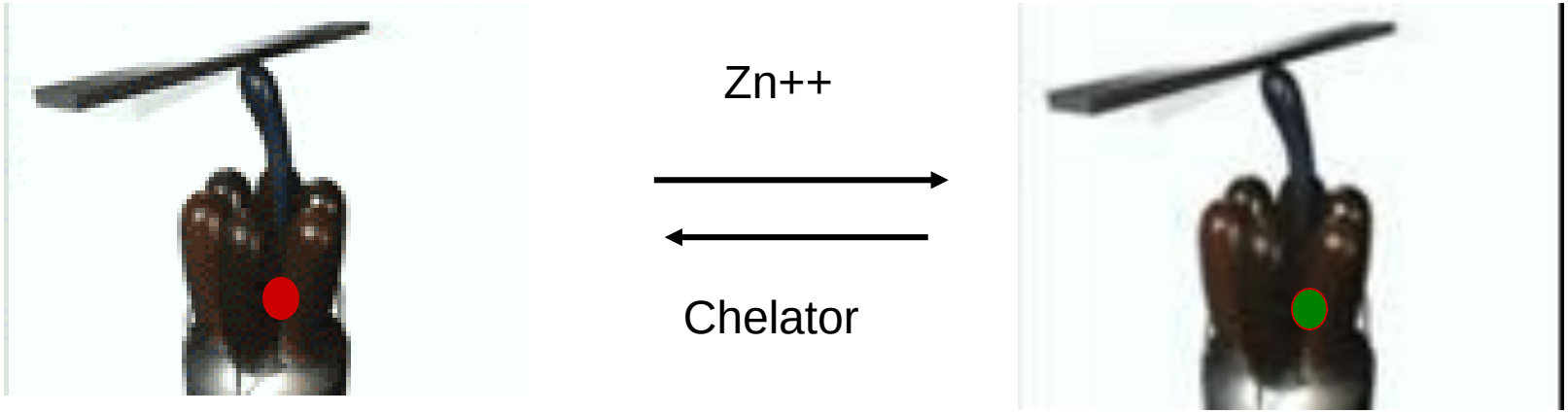
Now self assembly

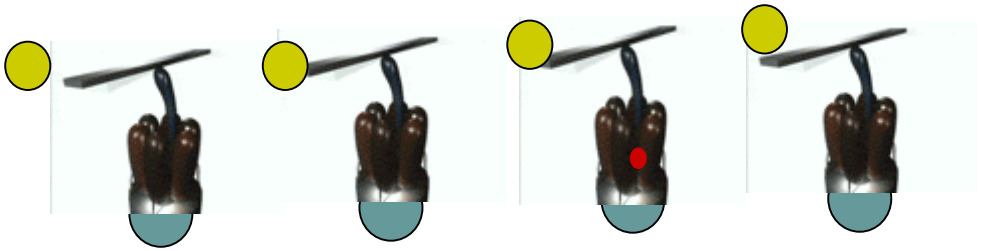
- ATP His binds Nickel, biotins bind avidin



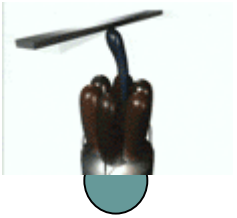
Attached ATPase to surface and propellers to ATPase

Specific Mutation that Inhibits in the presence of Zn++ determined computationally





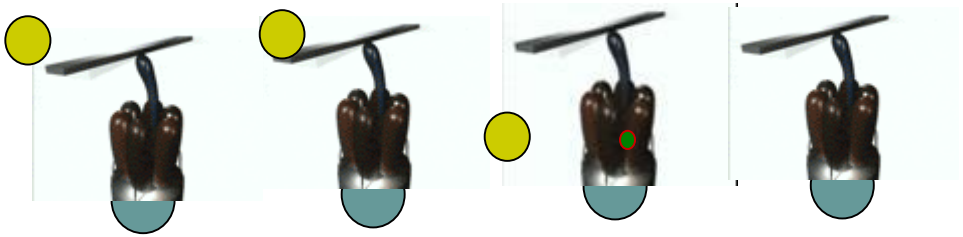
Output 1



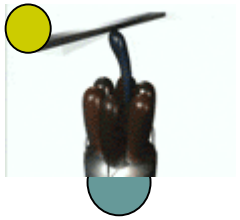
Output 2

Control

Zn^{++}

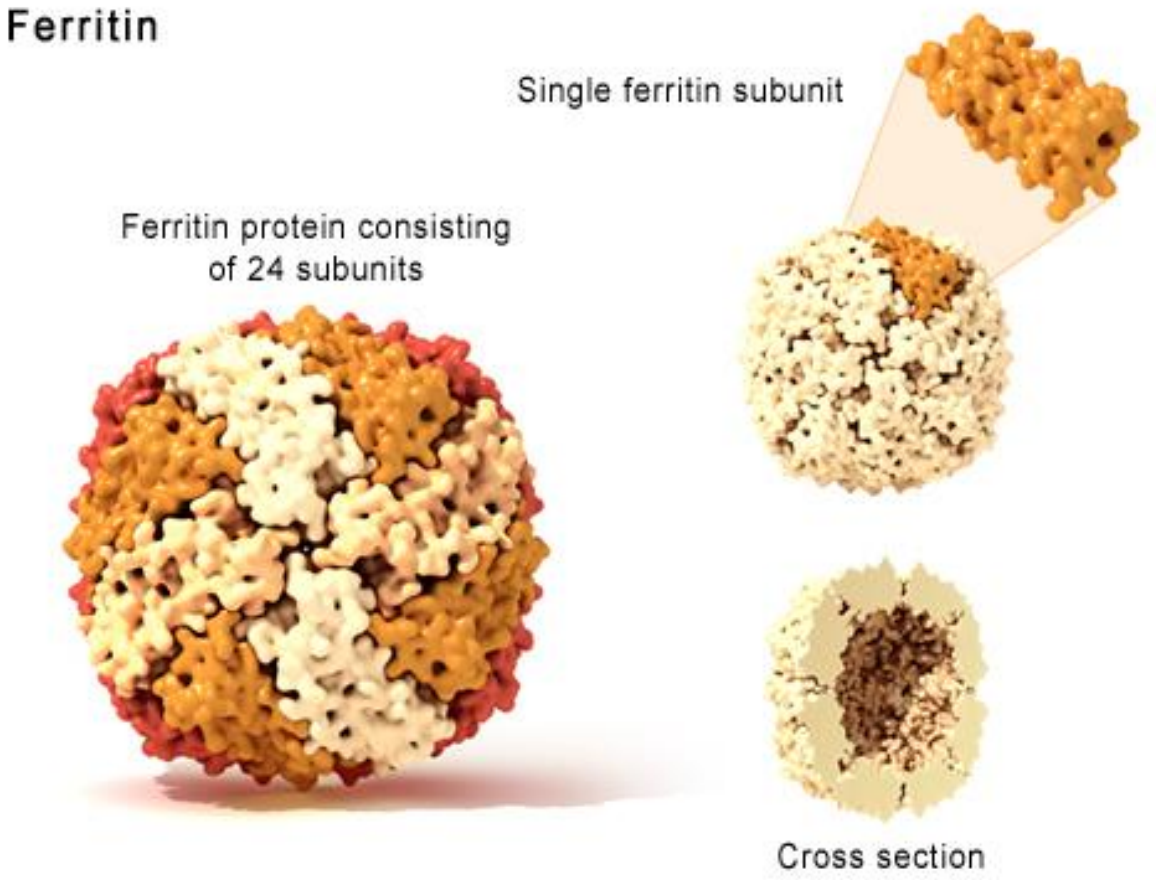


Output 1

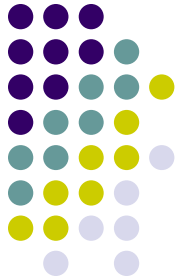


Output 2

Example 2: Ferritin as template for nanodots



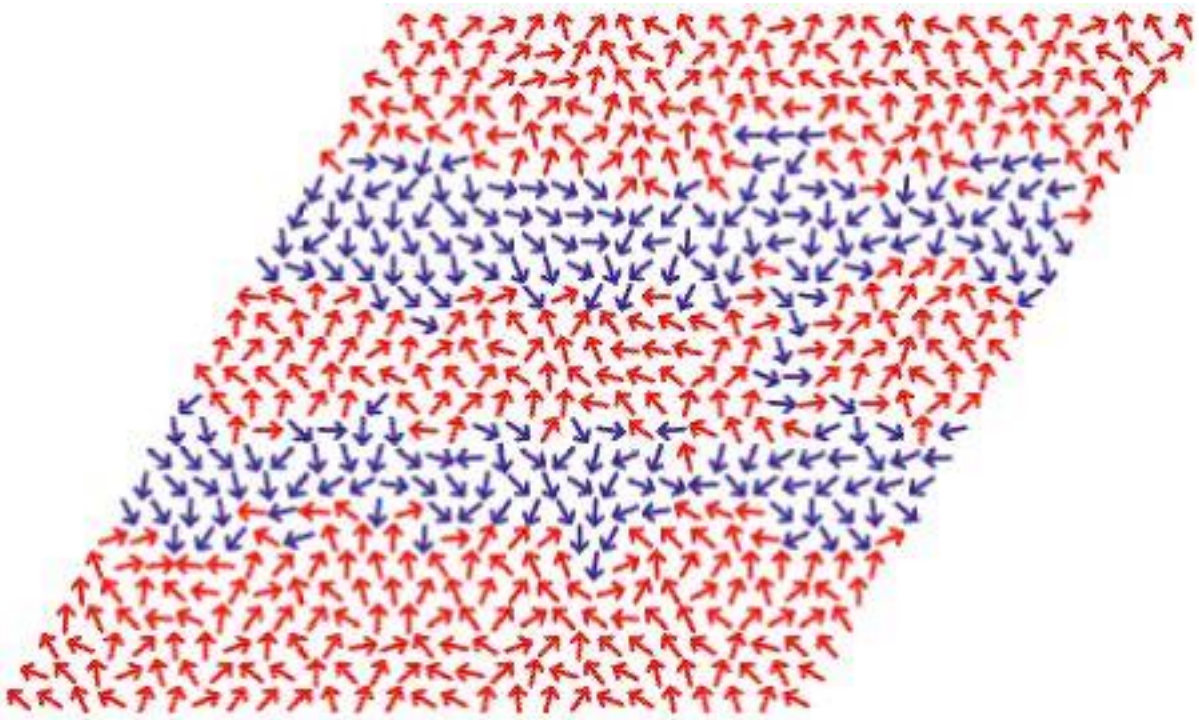
Background and Motivation



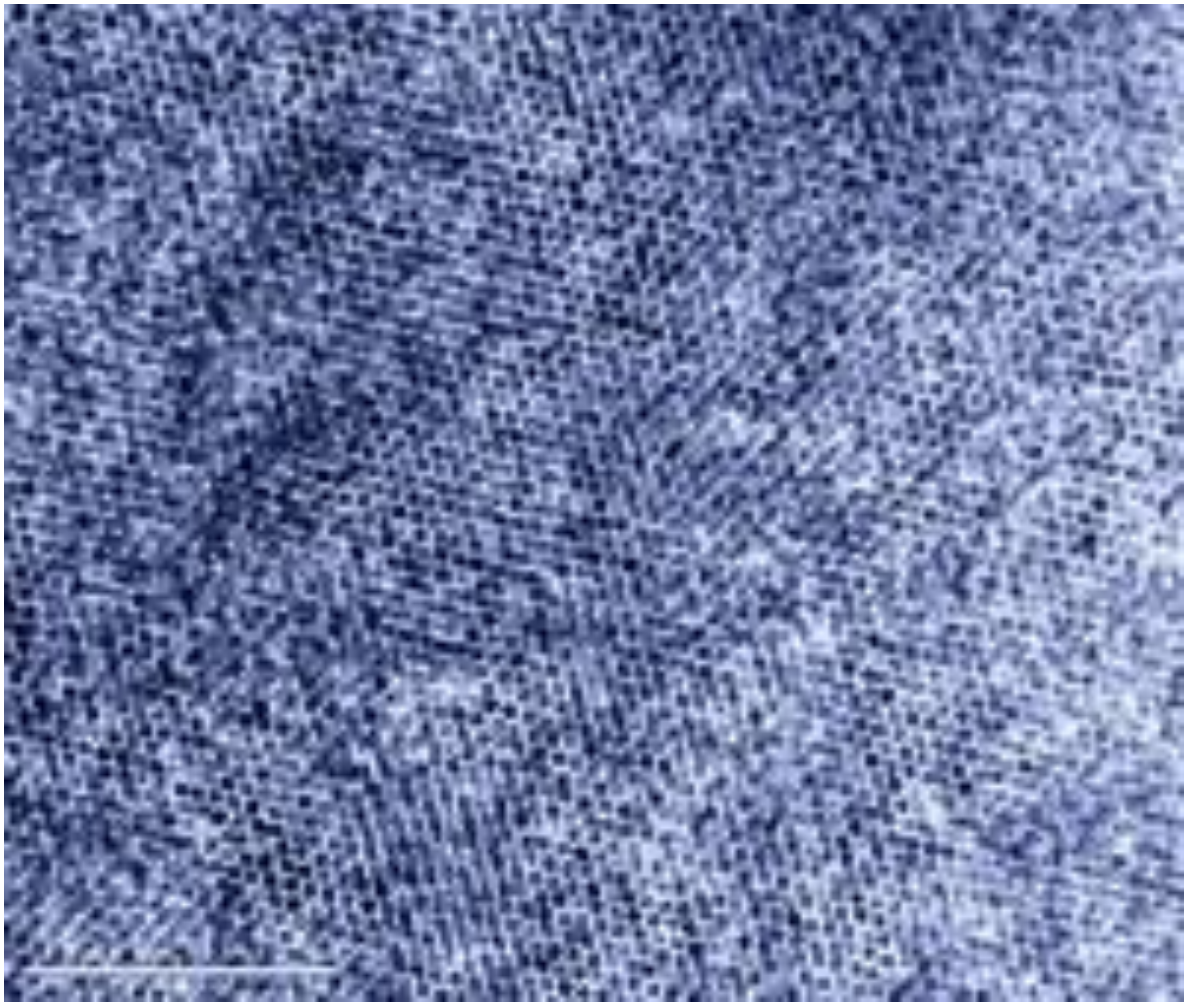
- Current hard drives 10-30 Gbits/in^2.
- Primary limitations derive from
  - Grain Size
  - Grain uniformity
  - Grain magnetic properties
- Overcoming these problems could lead to hard drives with 10Tbits/in^2.

http://www.nanomagnetics.com/

Movie or this….



Ordered ferritin array
Grain size 7nm



Lipids

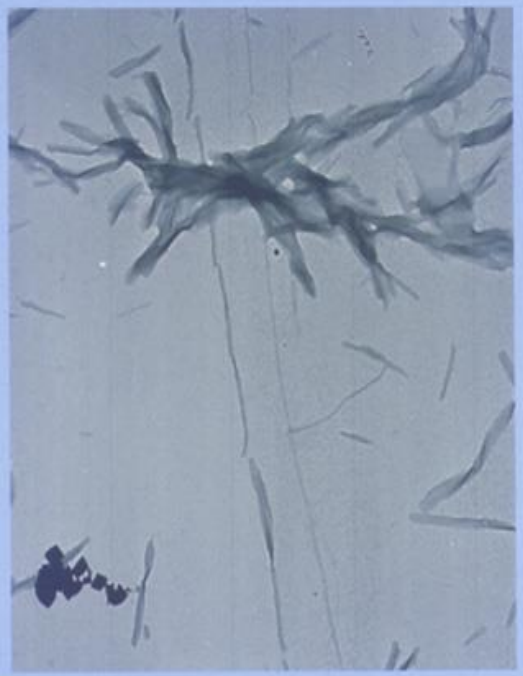
- Tubes, cubes, spheres etc
- Phase changes with temp, hydration, ionic strength
- Example lipid nanotubes

Nanoconduits

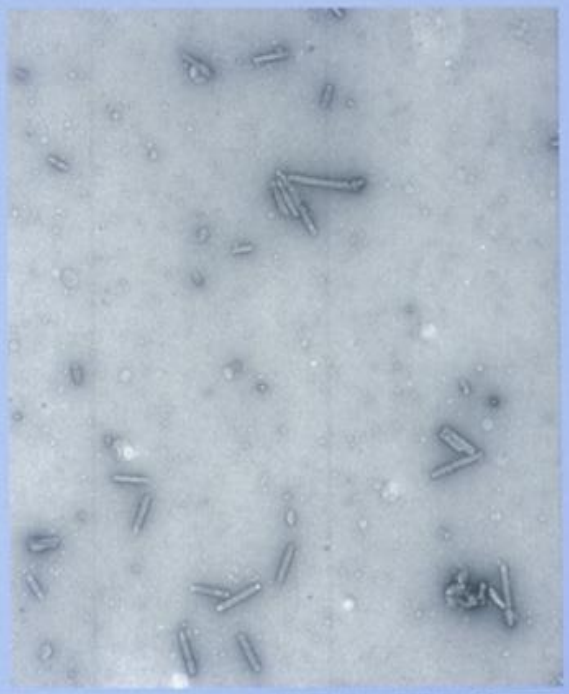
- Science, 273, 933 (1996) E. Evans, H. K. Bowman, A. Leung, D. Needham and D.A. Tirrell. "Biomembrane Templates for Nanoscale Conduits and Networks

Lipid Nanotubes

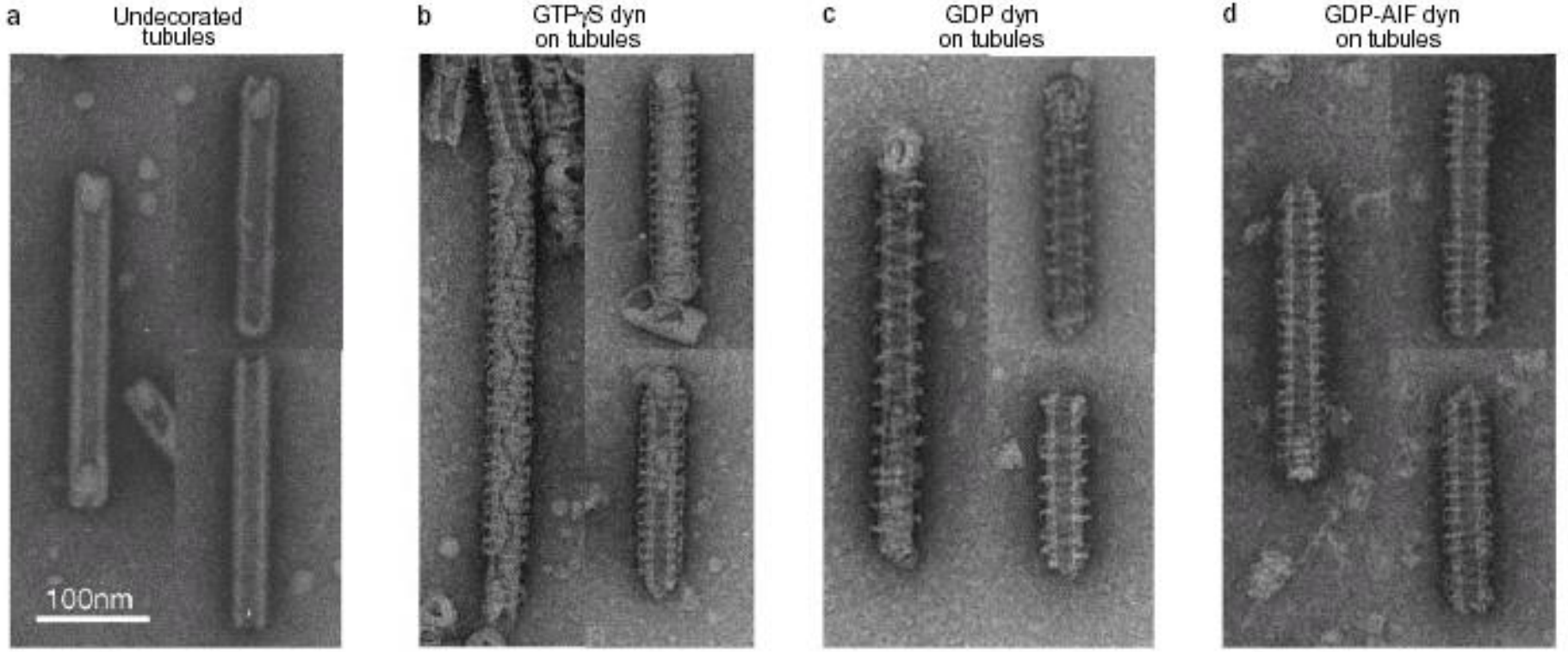
- 20-200 nanometers in diameter
- Can be 10's of microns in length
- Formed by a variety of methods
- Used as vehicles for drug delivery, similar to liposomes
- Model system for studying membrane associated proteins



1μm

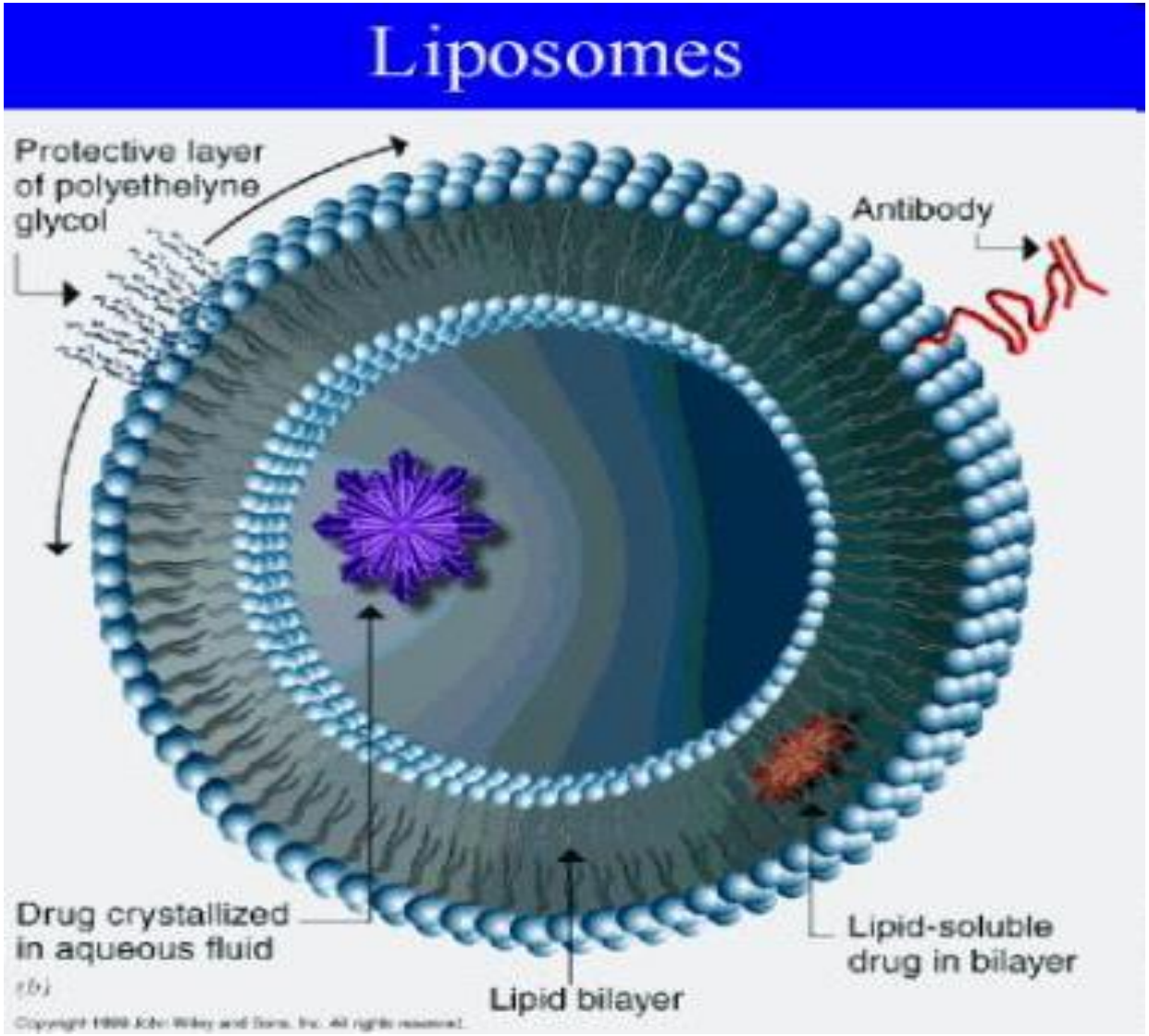


Lipid manipulation



Stowell, M.H., B. Marks, P. Wigge and H.T. McMahon (1999) **Nucleotide dependent conformational changes in dynamin: Evidence for a mechanochemical molecular spring**. Nature Cell. Biol. 1(1):27-32.

Liposome drug delivery



Protein Vector