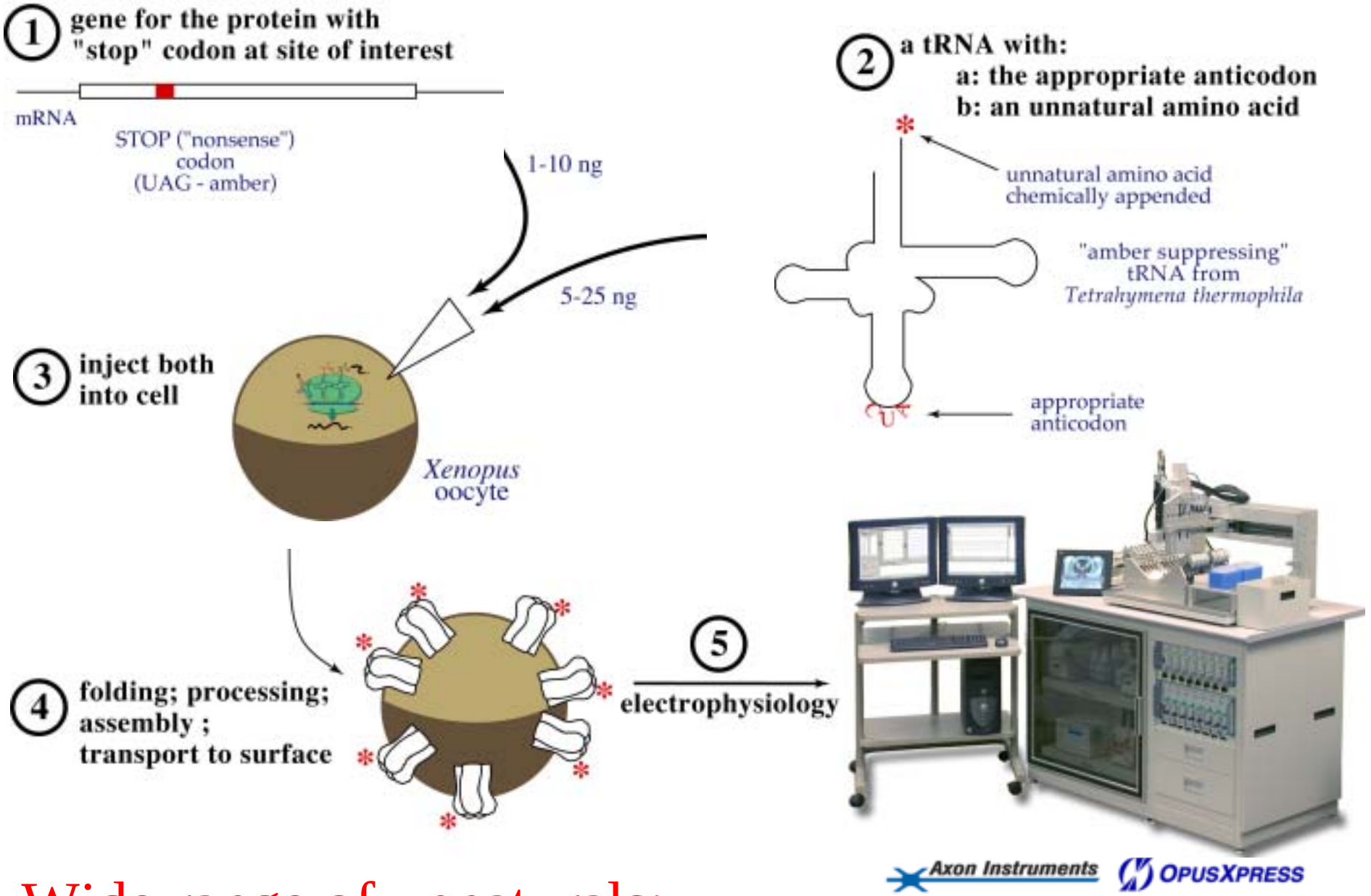
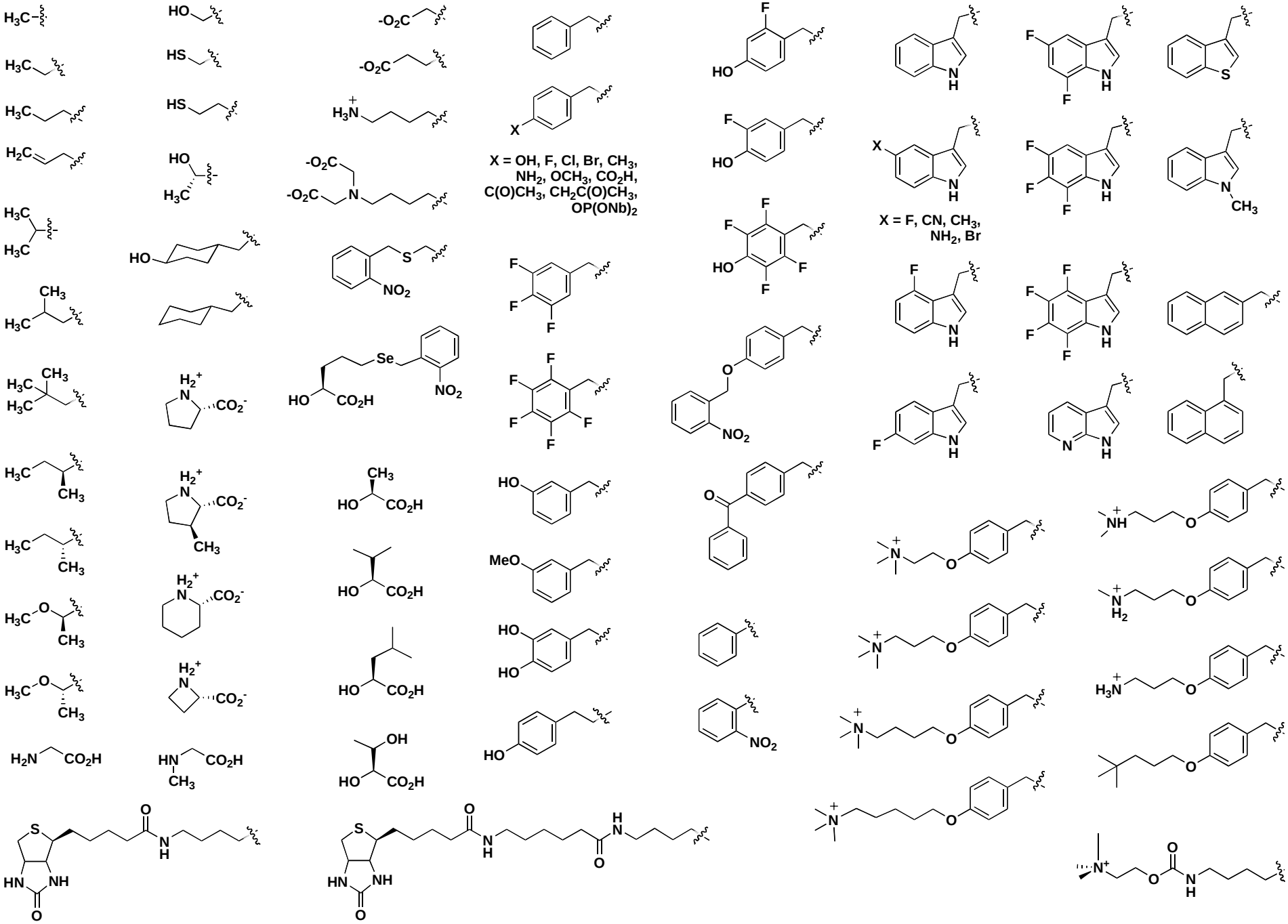
Structure-Function Studies

- Need a way to vary structure systematically
- Conventional site-directed mutagenesis is inadequate - the 20 natural amino acids provide limited structural diversity
- We vary structure using unnatural amino acid mutagenesis

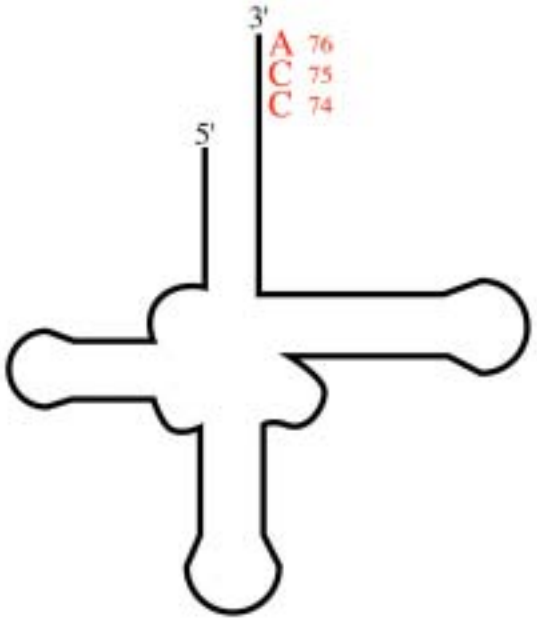
Unnatural Amino Acid Incorporation into Ion Channels Expressed in Oocytes



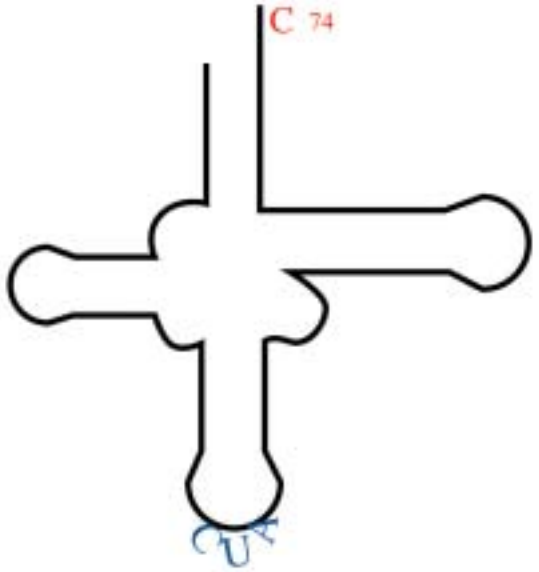
Wide range of unnaturals:


X = OH, F, Cl, Br, CH3, NH2, OCH3, CO2H, C(O)CH3, CH2C(O)CH3, OP(ONb)2
X = F, CN, CH3, NH2, Br

How do we do this?
tRNA Synthesis, Part A

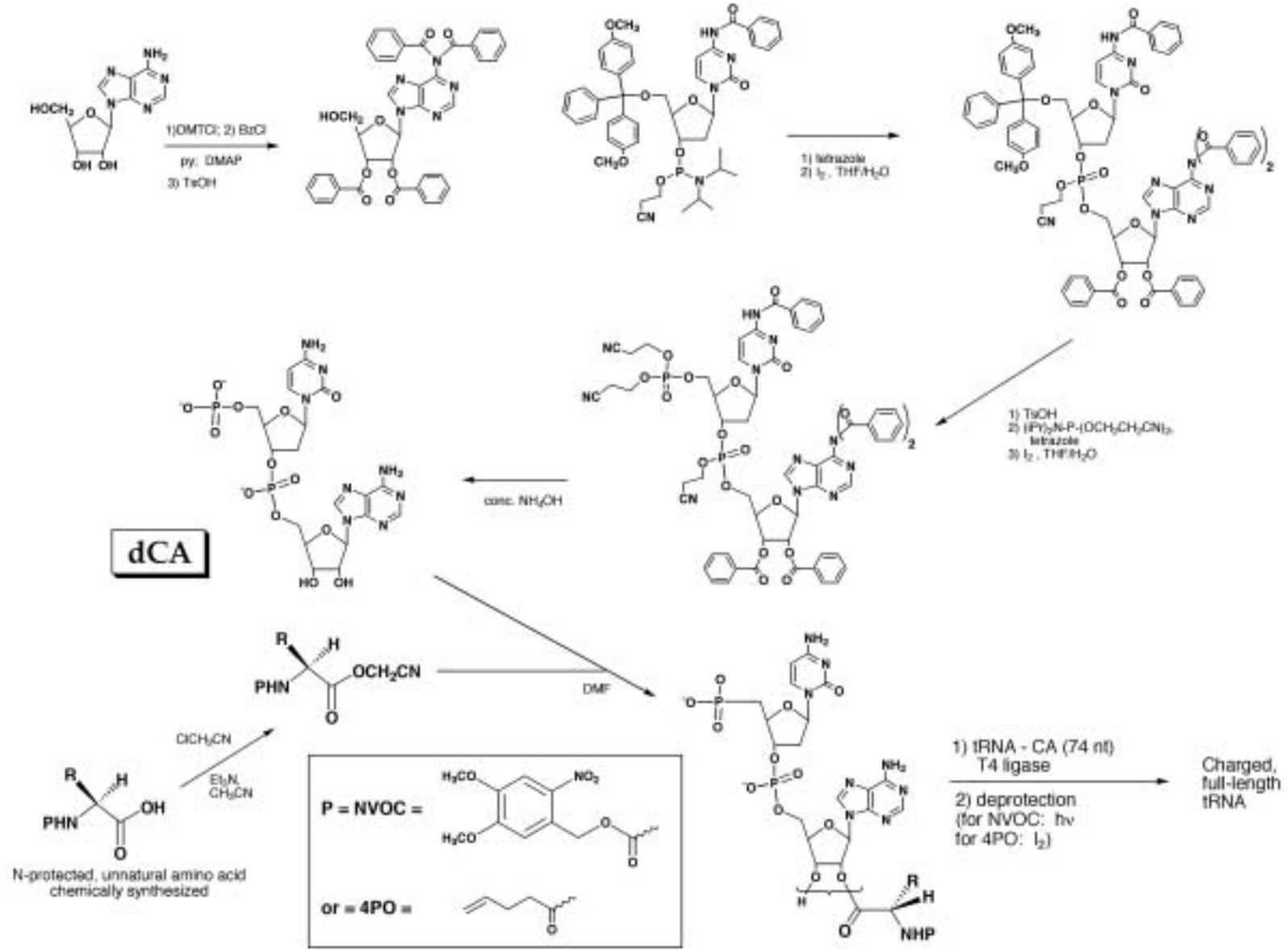


prototype tRNA is 76 bases, always ending in CCA

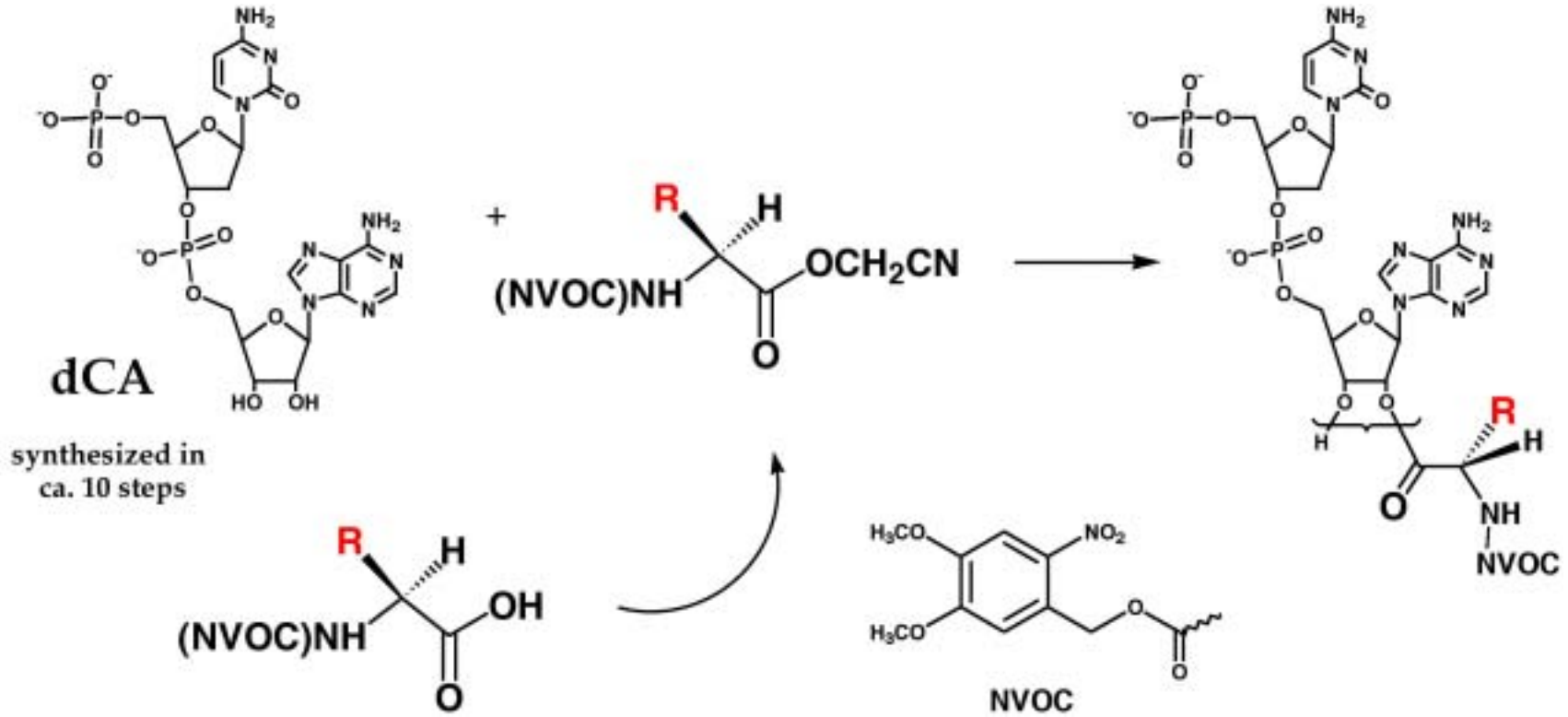


first 74 bases are made by transcription from a synthetic gene (DNA); necessary anticodon is designed in

The last two bases are chemically synthesized:



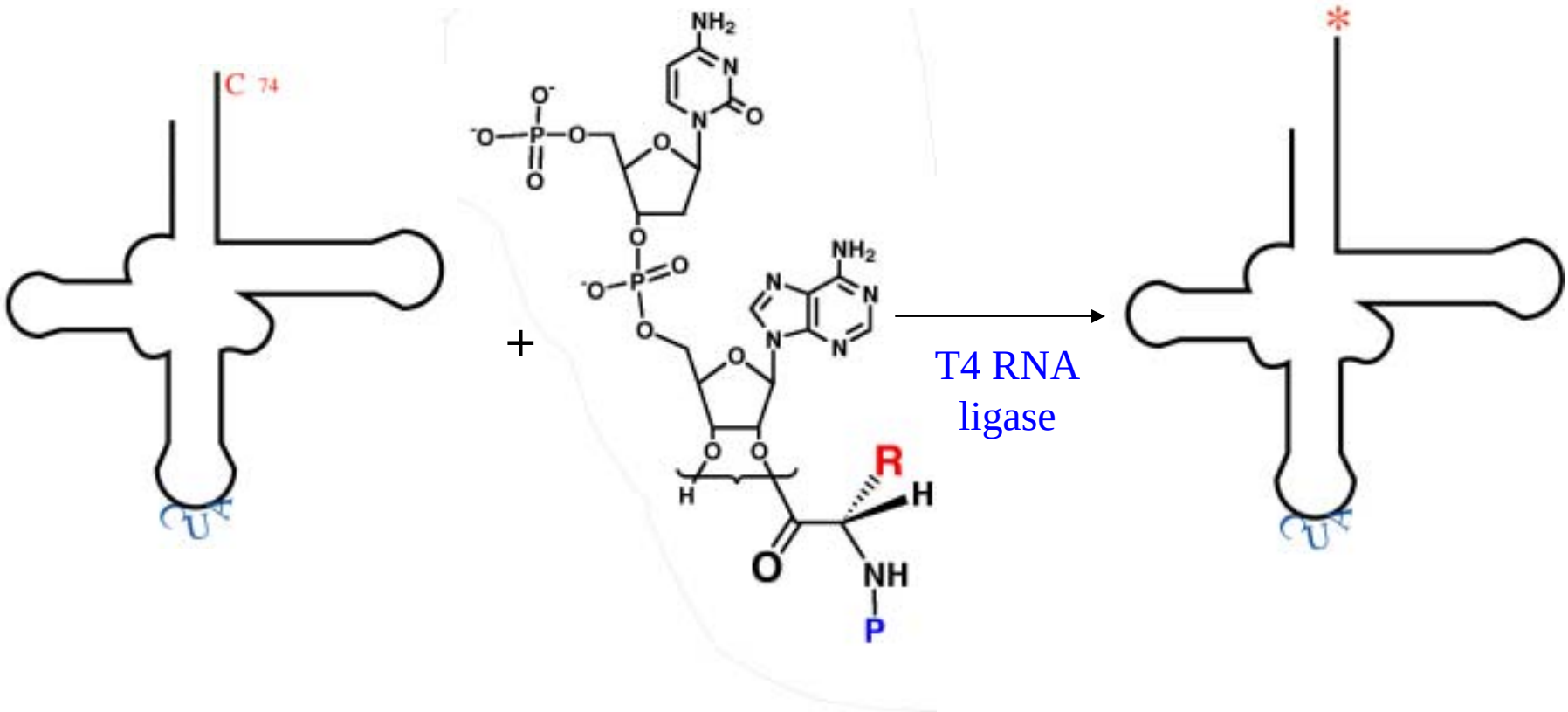
Synthesis of Unnatural Amino Acids



The unnatural amino acid

Also use 4-PO protecting group (previous slide); removed with I_2

Aminoacyl-tRNA Synthesis



Stored with amino group protected until just prior to injection

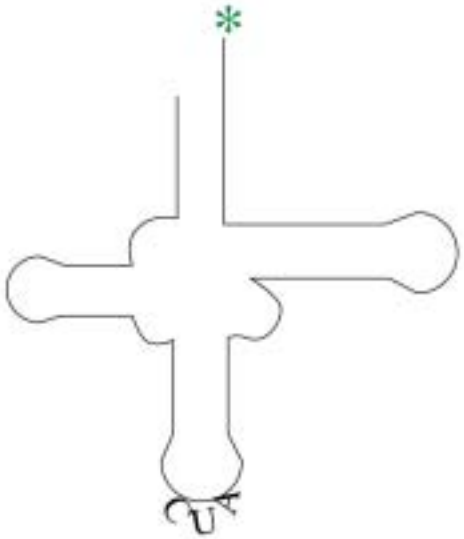
tRNA Design Issues

•the tRNA should be an efficient suppressor

•the tRNA must be ***orthogonal*** to the oocyte expresssion system

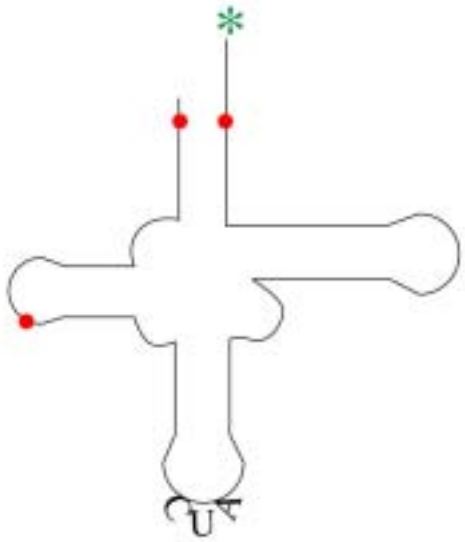
•not recognized by the endogenous aminoacyl-tRNA sythetases; i.e., not charge with a natural amino acid after it delivers the unnatural amino acid

tRNA DESIGN



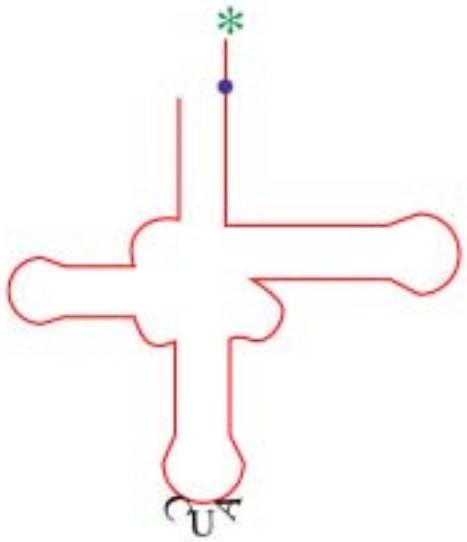
yeast tRNA-Phe
with amber suppressor anticodon
effective *in vitro* - Schultz (1989)

reacylated *in vivo*



MN3
new mutations introduced
to suppress reacylation
effective at several sites
of the nAChR *in vivo*

Nowak, et al. (1995)



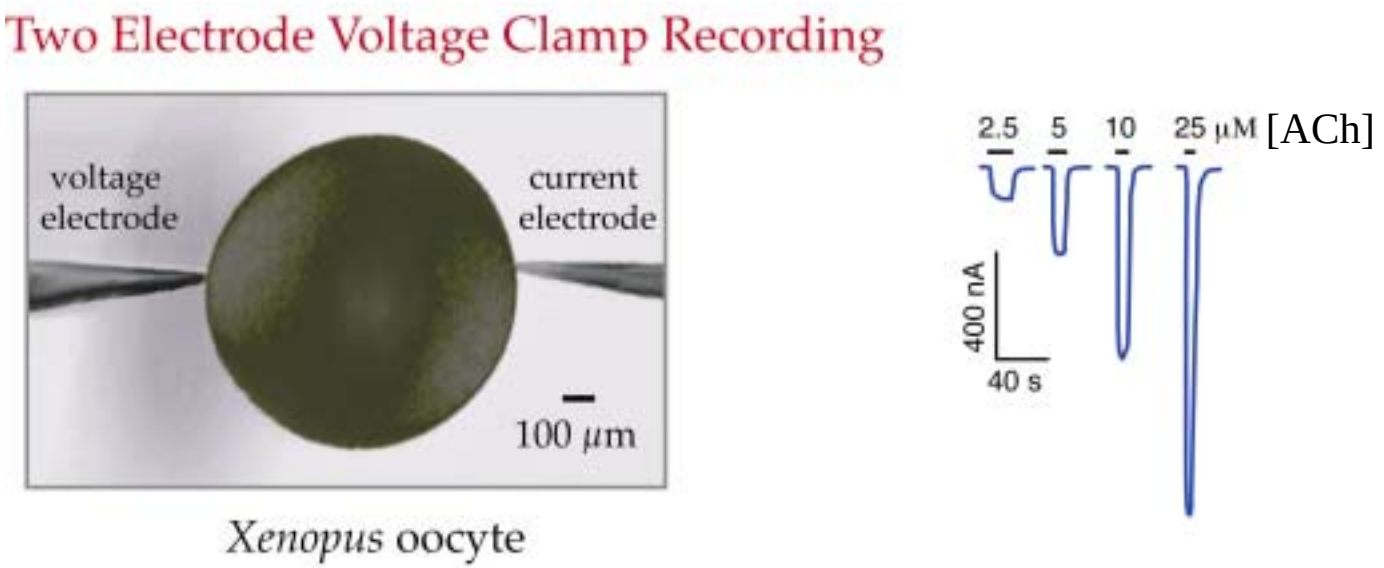
THG73
utilize non-standard genetic code of
Tetrahymena thermophila.
Natural amber suppressor (codes for Gln).
Much more efficient.
Nearly eliminates reaclyation problems.

Saks, Sampson et al., *JBC*, (1996)

Structure-Function Study

Flow of ions through a channel is equivalent to electrical current

Functional Probe: Electrophysiology



Simply inject DNA or mRNA into oocyte, return 24 hours later.

Ligand-gated ion channel is expressed and it responds to neurotransmitter (eg., ACh) by generating a current

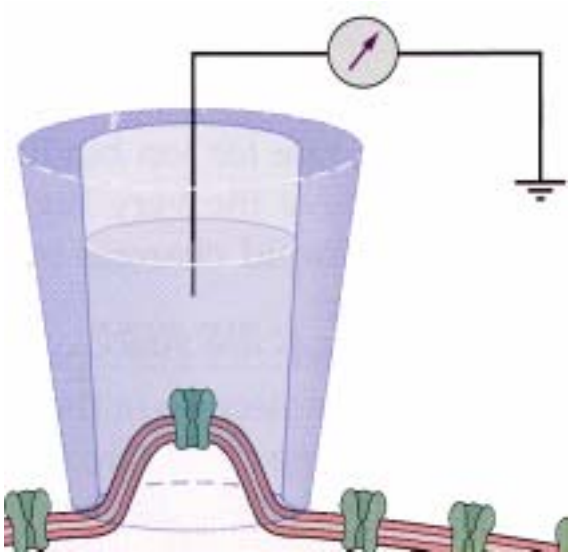
Physiology and pharmacology same as in natural environment.

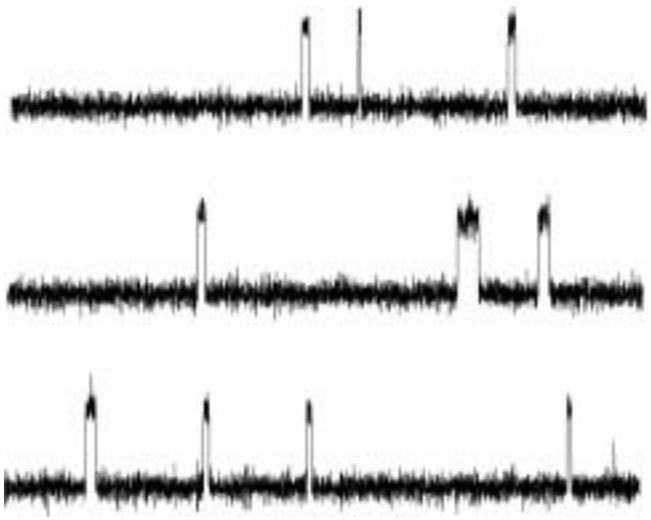
Structure-Function Study

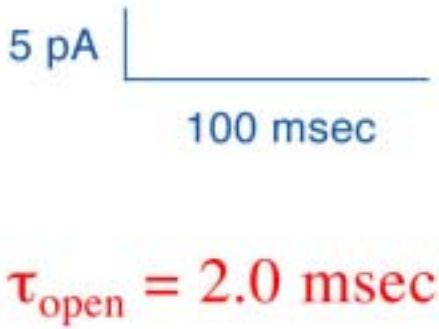
Single molecule recording via the patch clamp

Functional Probe: Electrophysiology

The Patch Clamp







Single molecule kinetics in real time

A Wide Range of Channels & Receptors

- Nicotinic Acetylcholine Receptor

  α, β, γ, δ, of muscle; $\alpha 4$, $\alpha 7$ of neuronal

- 5-HT_3 Receptor
- NMDA Receptor
- GPCRs
- Transporter - GAT1
- K^+ Channels

  voltage gated (Shaker, Shaker-IR)

  inward rectifying (Kir 1.1, 2.1, 3.1, 3.4)
- Na^+ Channels
- CFTR

Any protein amenable to efficient heterologous expression