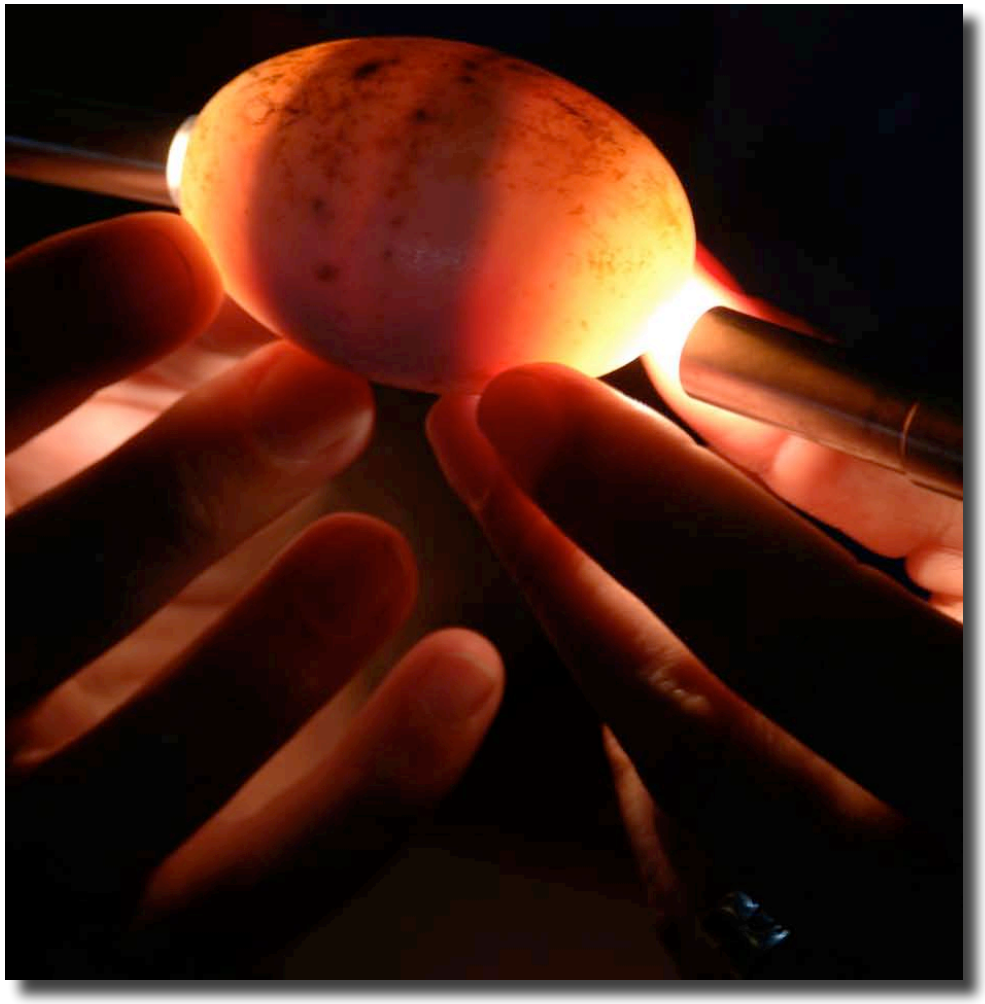
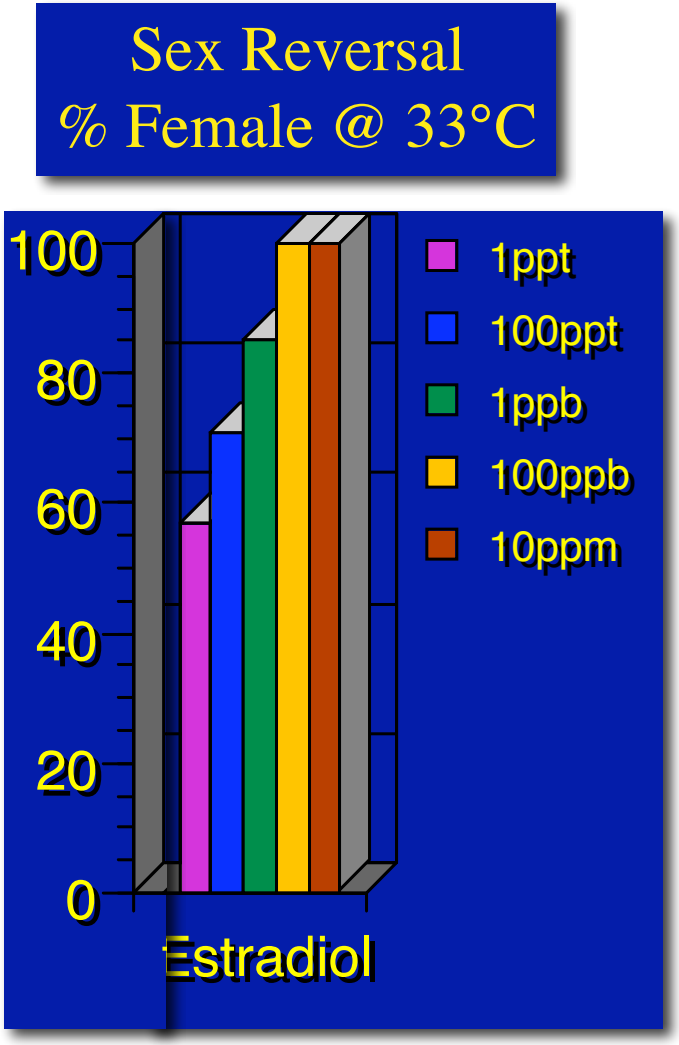
Techniques in Reproductive Biology

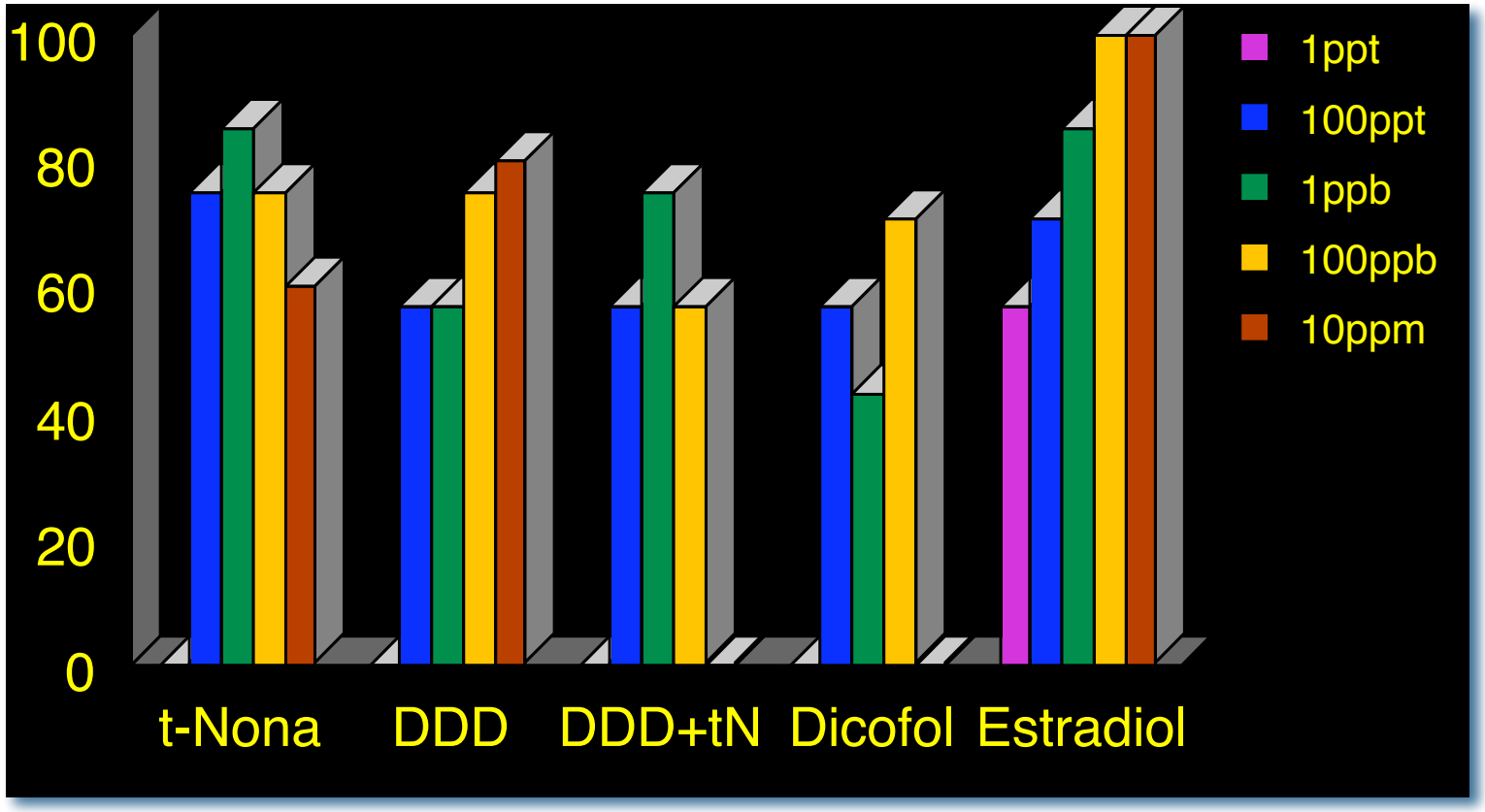


Bioassay

- Use of a known biological response
- Develop a dose response curve
- Access unknowns
- Still extensively used



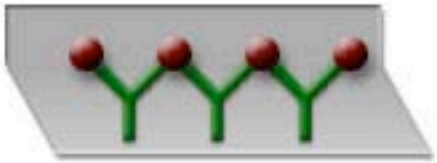
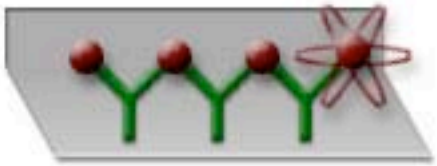
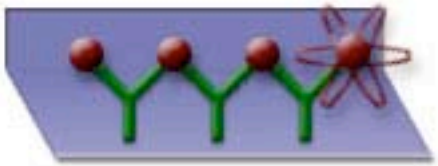
Alligator Sex Reversal



All compounds estrogenic - but not ‘complete estrogen’

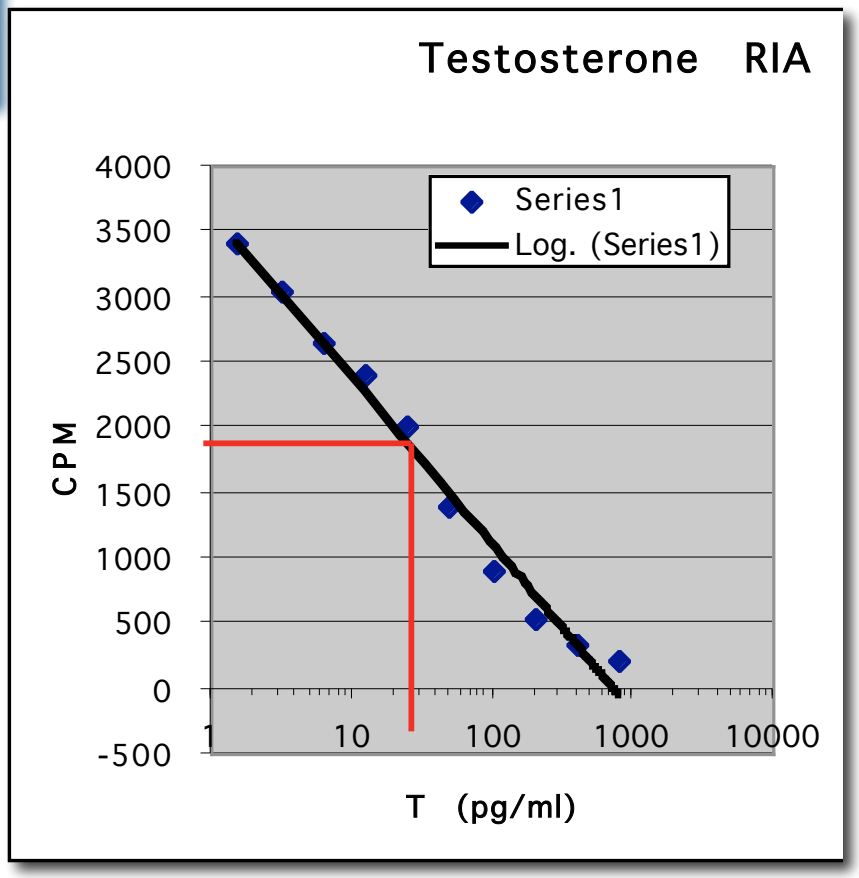
Radioimmunoassay

- Used to measure quantitatively hormone concentrations in blood, receptors in tissue, etc.
- A competitive binding assay using
  - Radio = radioactive label
  - Immuno = specific antibody
  - Assay = quantitative approach

REAGENTS:	POSITIVE SAMPLE	NEGATIVE SAMPLE
Ab specific for hormone (coating the filter) Unknown sample with hormone Allow time to react Wash away unbound substances	high level of hormone 	low level of hormone 
REAGENTS: ^{125}I-labeled hormone Allow time to react Wash away unbound radiolabeled hormone	 	
PROCEDURE: measure radioactivity in a gamma counter RESULT: amount of radioactivity is inversely proportional to the concentration of hormone in the sample.		

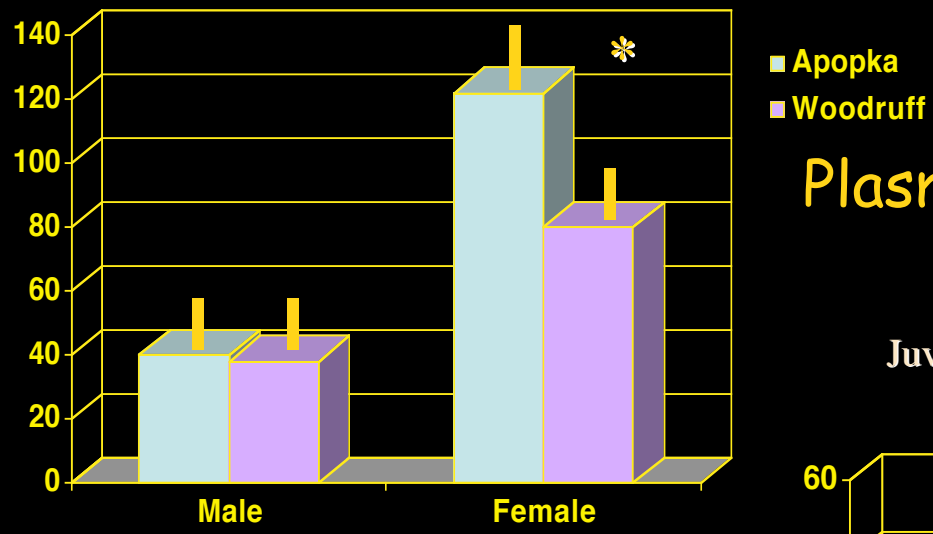
				CPM 1	CPM 2	MEAN CPM
				CPM 1	CPM2	
TC				14595	15187	14891
NSB				87	96	92
B0		0		2919	2898	2909
STD	1	1.5625		2862	2868	2865
STD	2	3.125		2750	2719	2735
STD	3	6.25		2614	2672	2643
STD	4	12.5		2352	2434	2393
STD	5	25		2014	1980	1997
STD	6	50		1414	1388	1401
STD	7	100		935	869	902
STD	8	200		535	511	523
STD	9	400		308	339	324
STD	10	800		209	206	208

- 1900 cpm = 28 pg/tube
- If:
  - 100 µl/tube
- Then
  - Concentration in ml
  - 10 X 28 pg/tube
  - 280 pg/ml



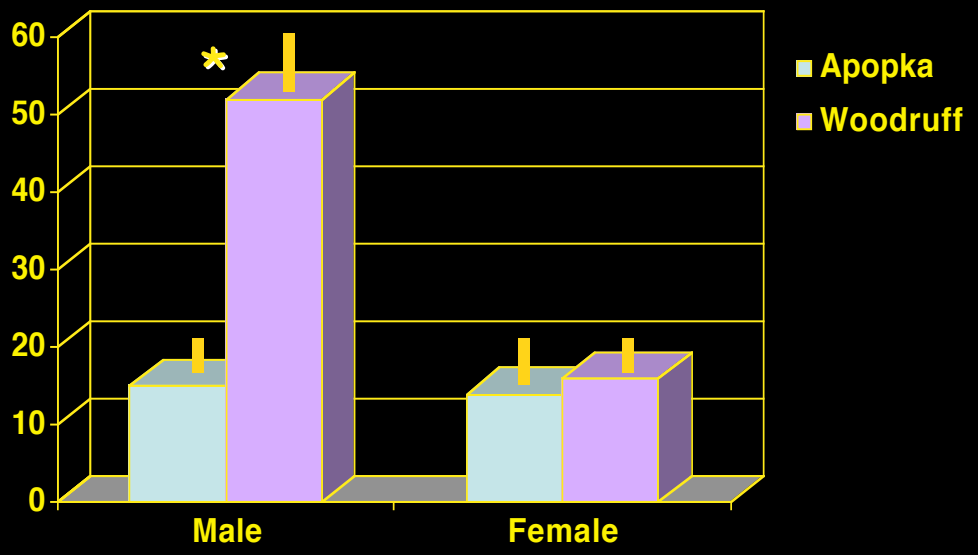
Plasma Estradiol (pg/ml)

Juvenile Alligators - 9 mo old



Plasma Testosterone (pg/ml)

Juvenile Alligators - 9 mo old



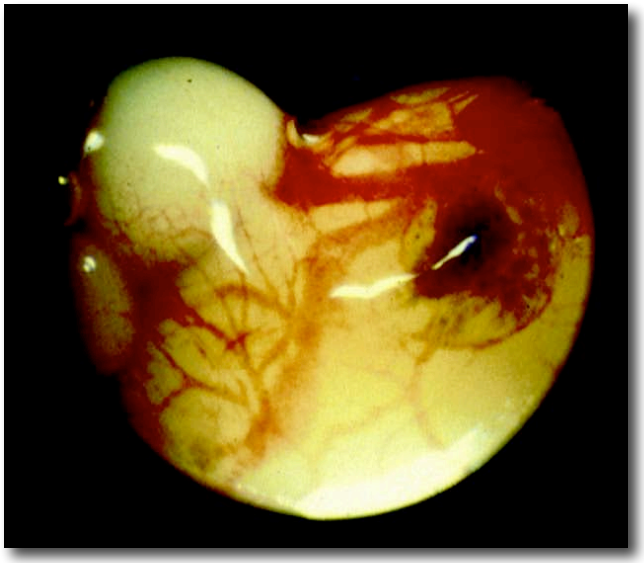
Guillette et al. (1994) Environ. Health Perspec. 102:680-688

Surgery

- Earliest technique
  - 1) "Extirpation"
    - Remove tissue
  - 2) See what happens
  - 3) Replacement
  - Still used extensively
  - Interpretation can be difficult

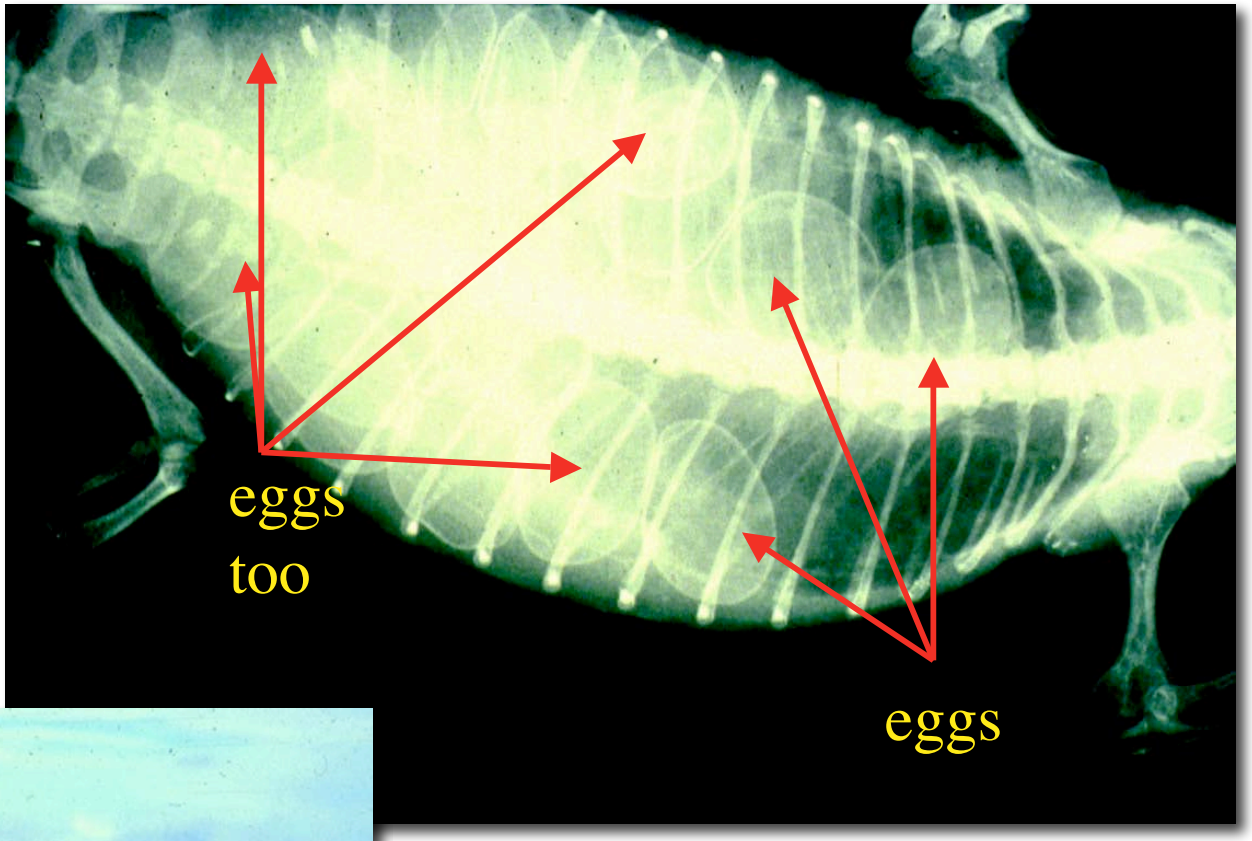
X-Ray & Other Imaging

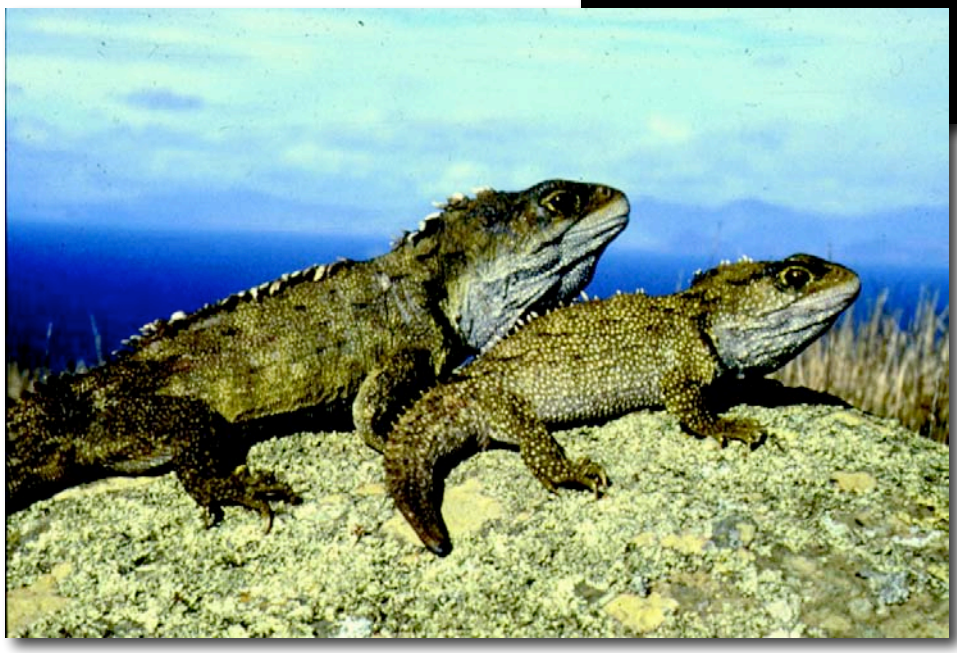
- Modern imaging technology extensive
  - X-ray; MRI; CAT scan
- Used for non- to minimally invasive 'view'
- Can provide extensive information



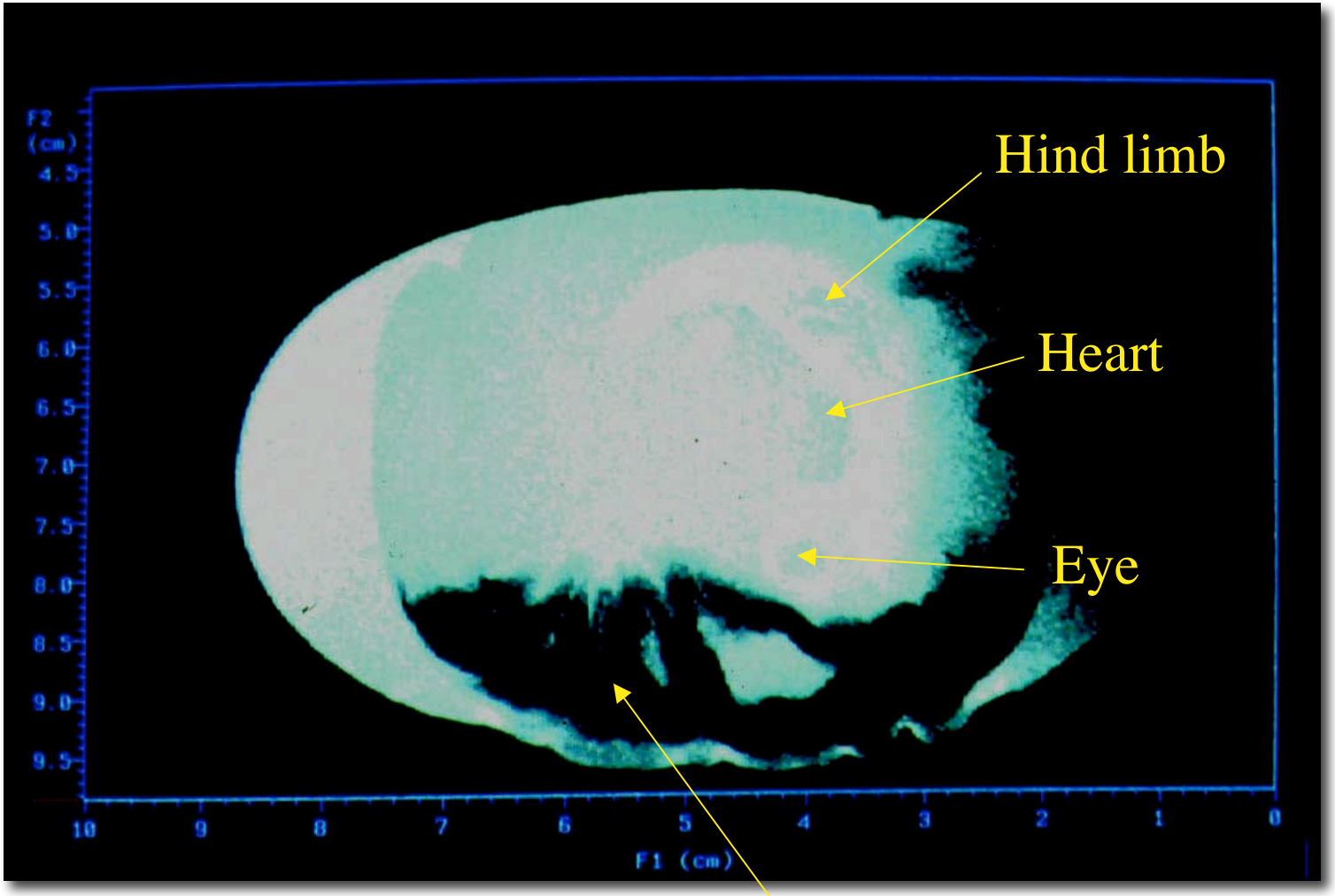
Laparoscopy of Tuatara ovary

X-ray of gravid tuatara



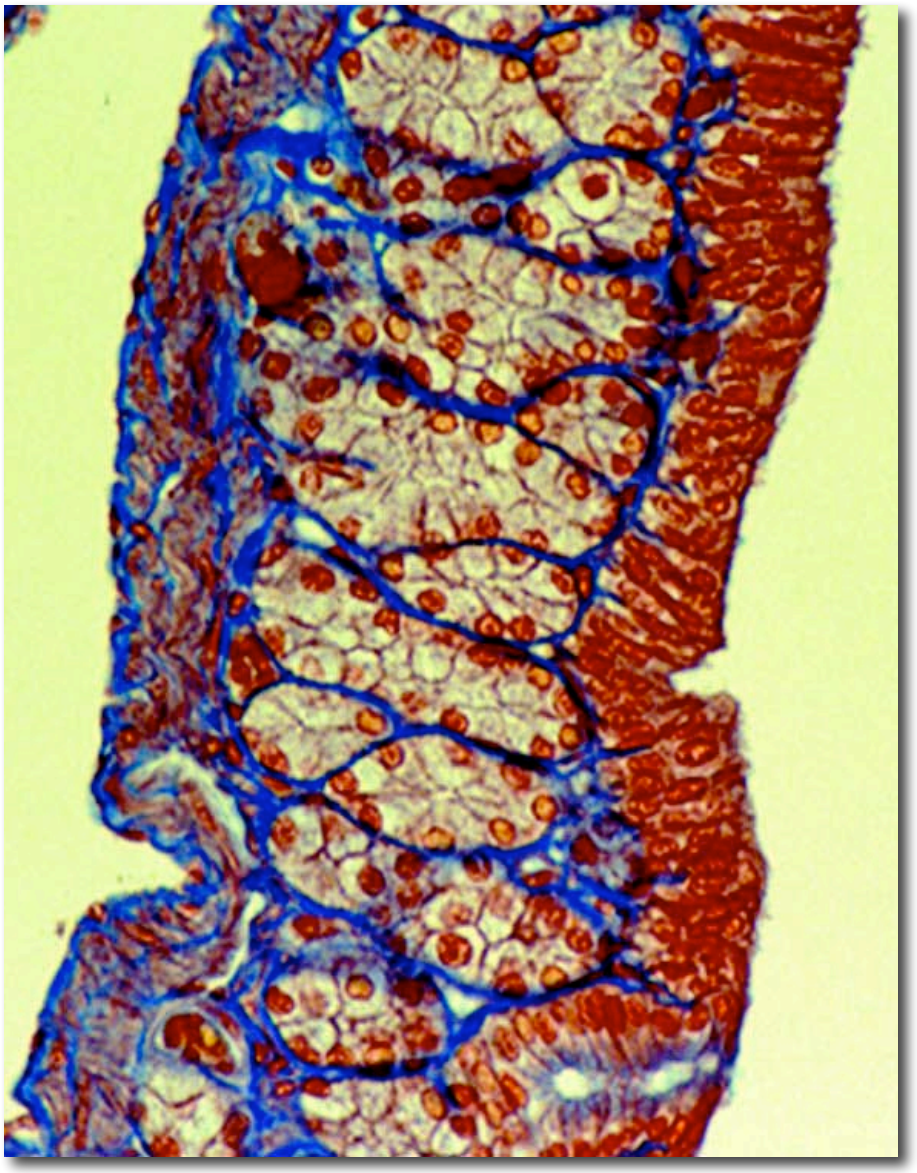


MRI of an Alligator egg



Histology

- Sections of tissues cut at micrometer thickness or smaller
- Provides view of tissue at cellular or subcellular level
- Initial process of many other processes



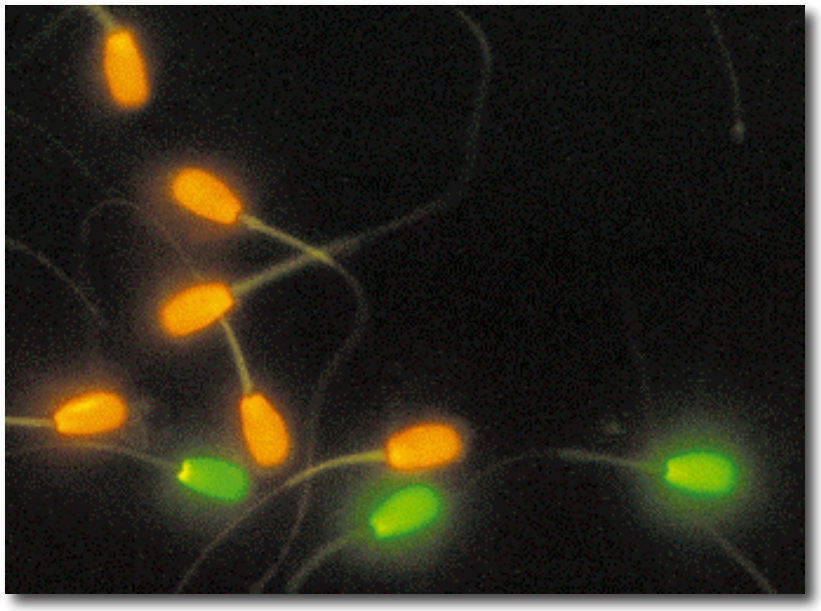
SEM/TEM

- Electron microscopy allows sub cellular view of cells
- Powerful tools for anatomy and physiology
- SEM: tissue dried - coated with gold
- TEM: tissue fixed - cut at < 1 μm

SEM of uterine surface with
Egg shell fibers extruding

Histochemistry

- Used to examine specific enzymatic reactions of cells
  - Can be used to identify pathological tissues
  - Or live and dead tissue

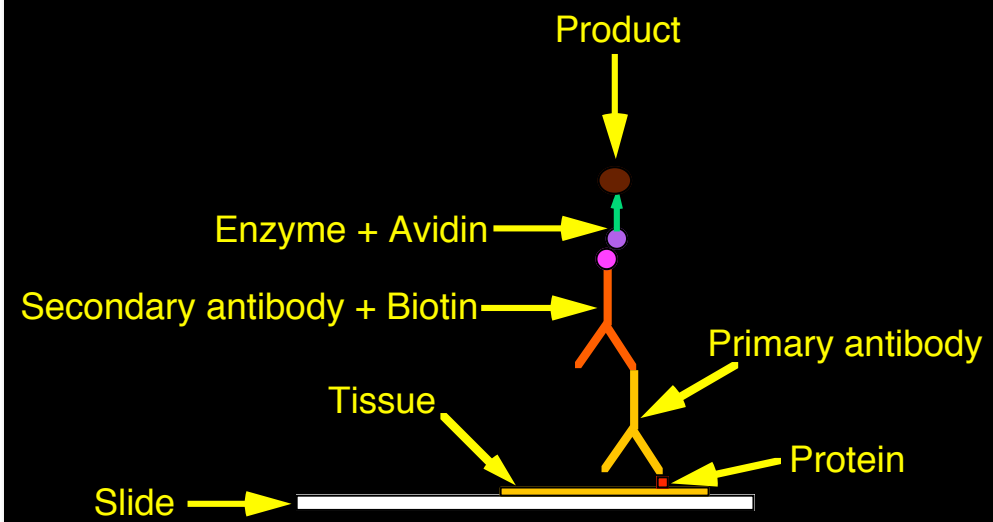


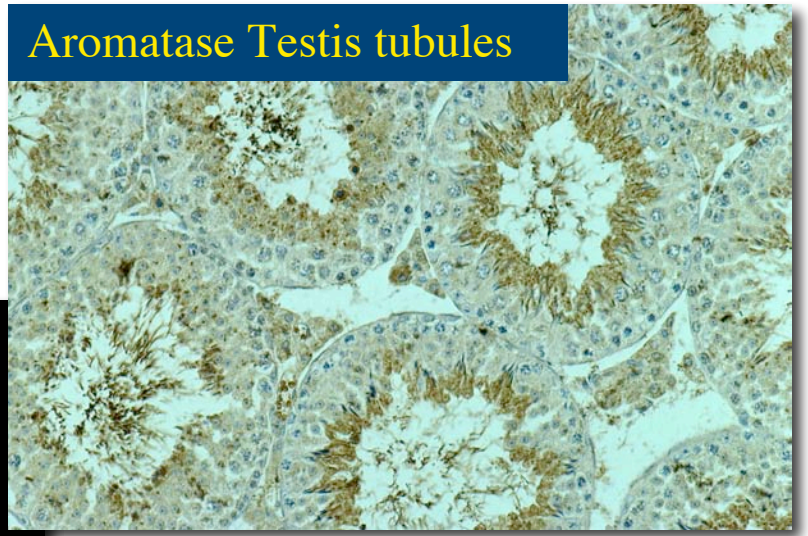
- Bull sperm
  - live - green
  - dead - red

Immunocytochemistry

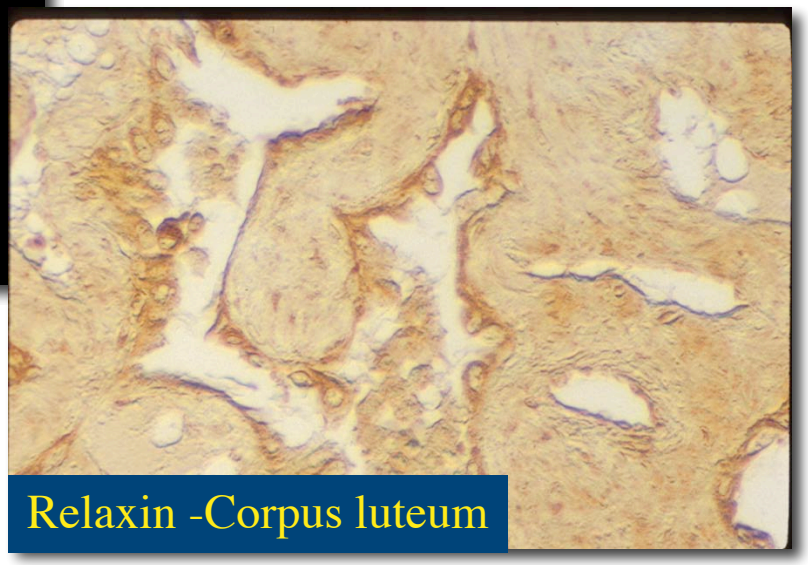
- Used to identify ***location*** of protein in a cell/tissue
- Use of a specific antibody and targeted enzyme reaction

Immunocytochemistry

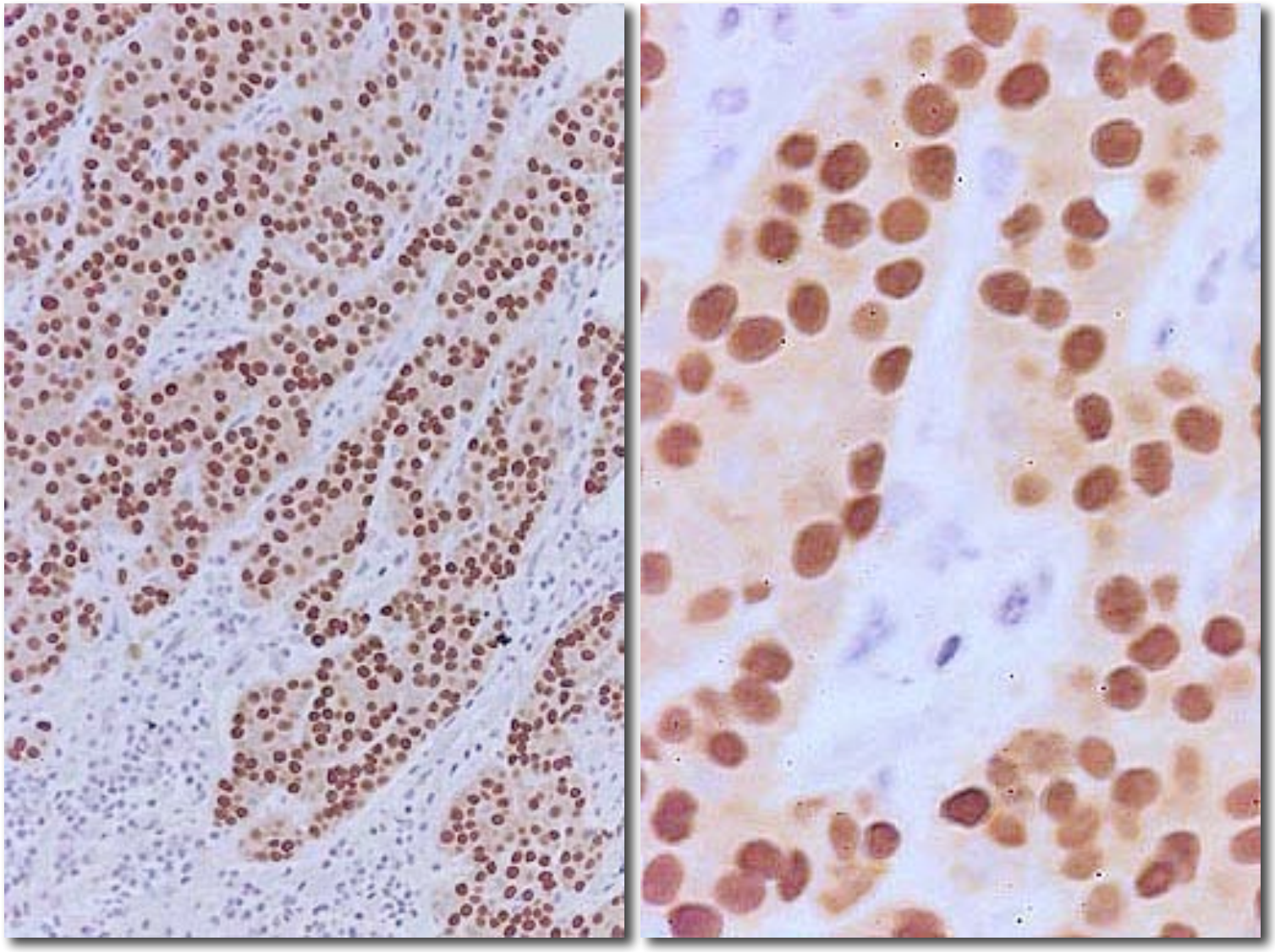




Aromatase Testis tubules



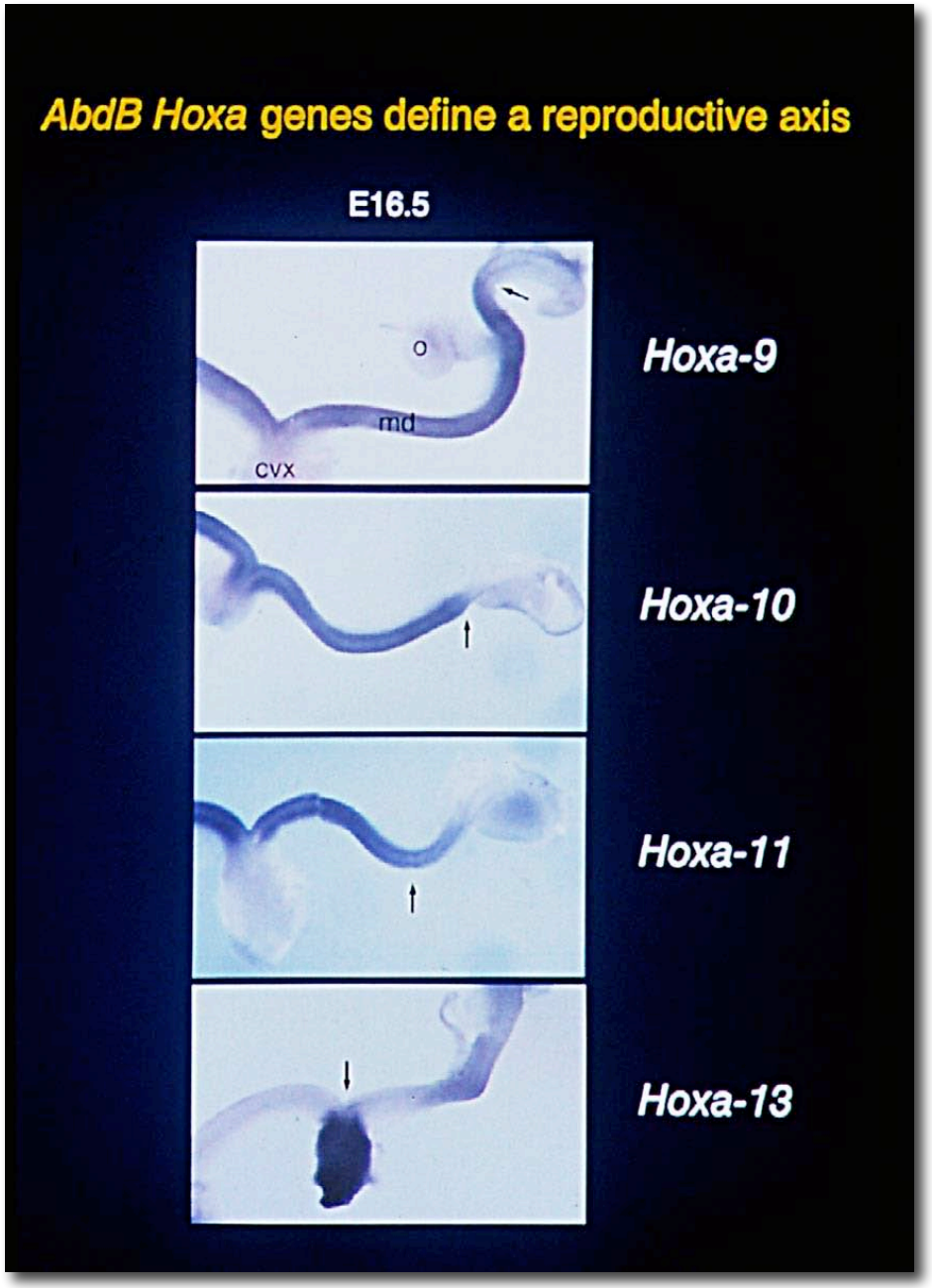
Relaxin -Corpus luteum



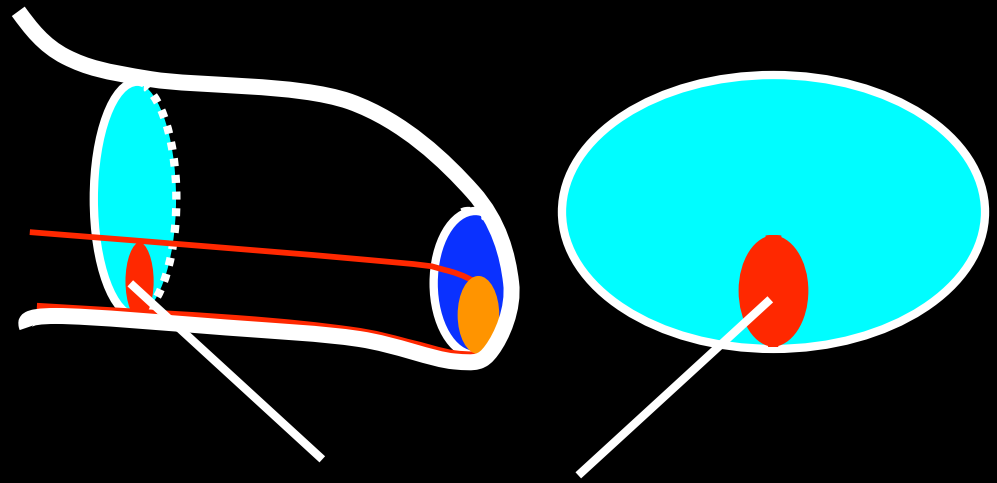
ICC for ER in Uterine tissue

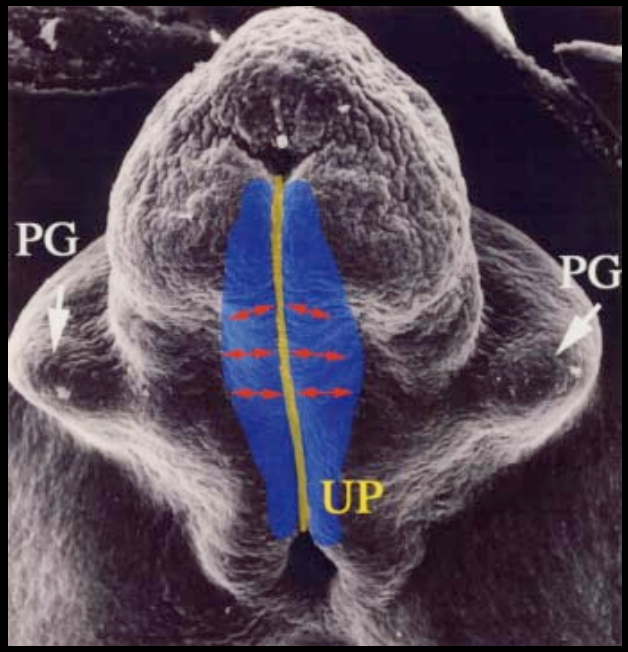
In situ Hybridization

- Used to localize specific mRNA in tissue or cell
- Targeted probe complementary to the mRNA



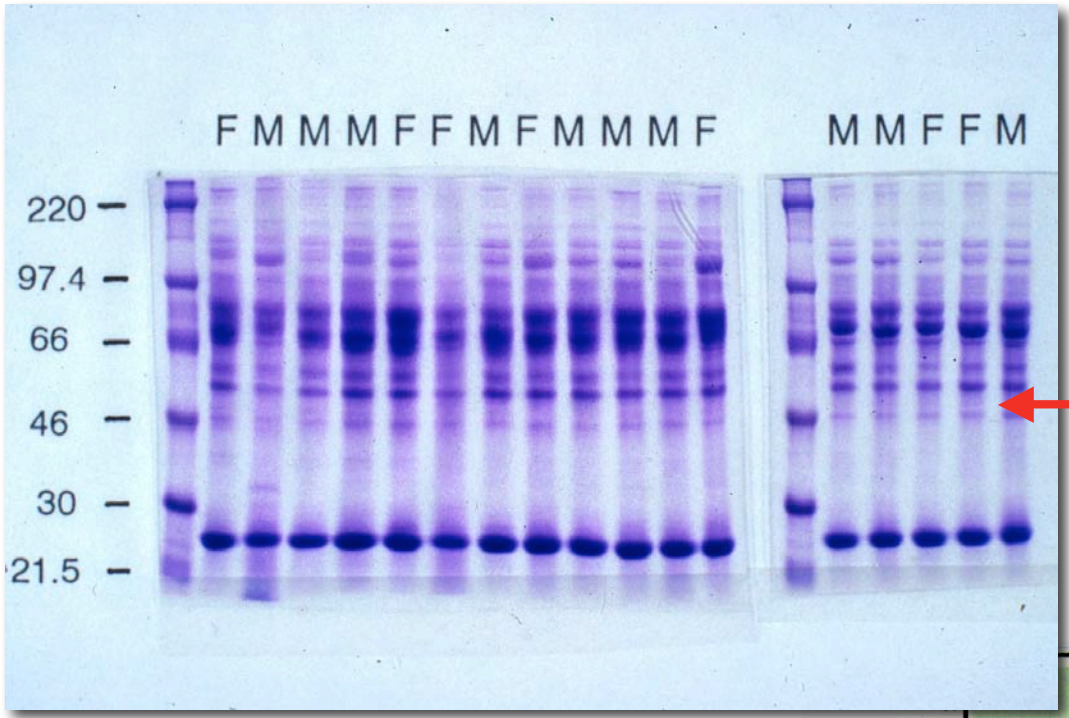
Gene expression pattern during genital tubercle development



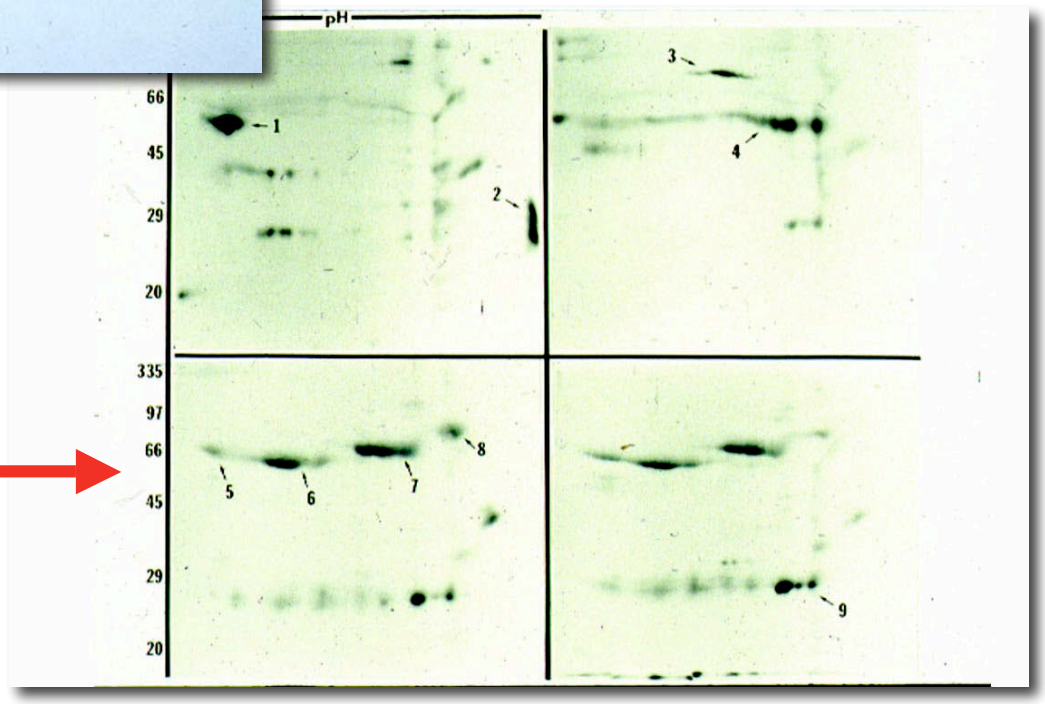


Electrophoresis

- Use molecular weight &/or charge to separate chemicals
  - proteins or RNA or DNA commonly isolated using electrophoresis
  - 1 or 2 dimensions



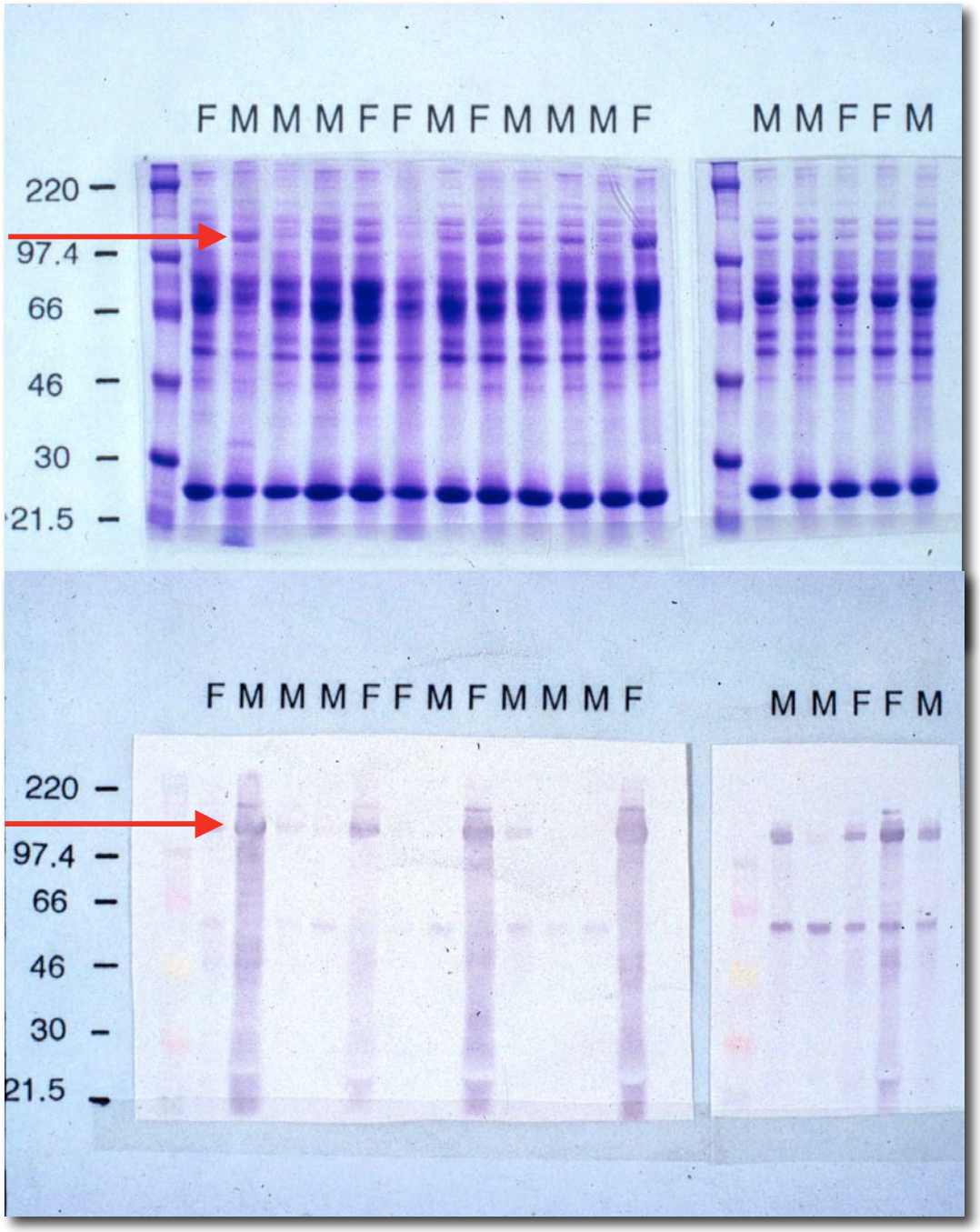
1D-SDS PAGE for
Serum proteins in fish



2D-SDS PAGE for
Oviduct proteins in gator

Western blots

- Protein from electrophoretic gel transferred to membrane
- 'Stained' with a specific antibody
  - ICC on gel blot
- Identifies a specific band associated with a specific protein



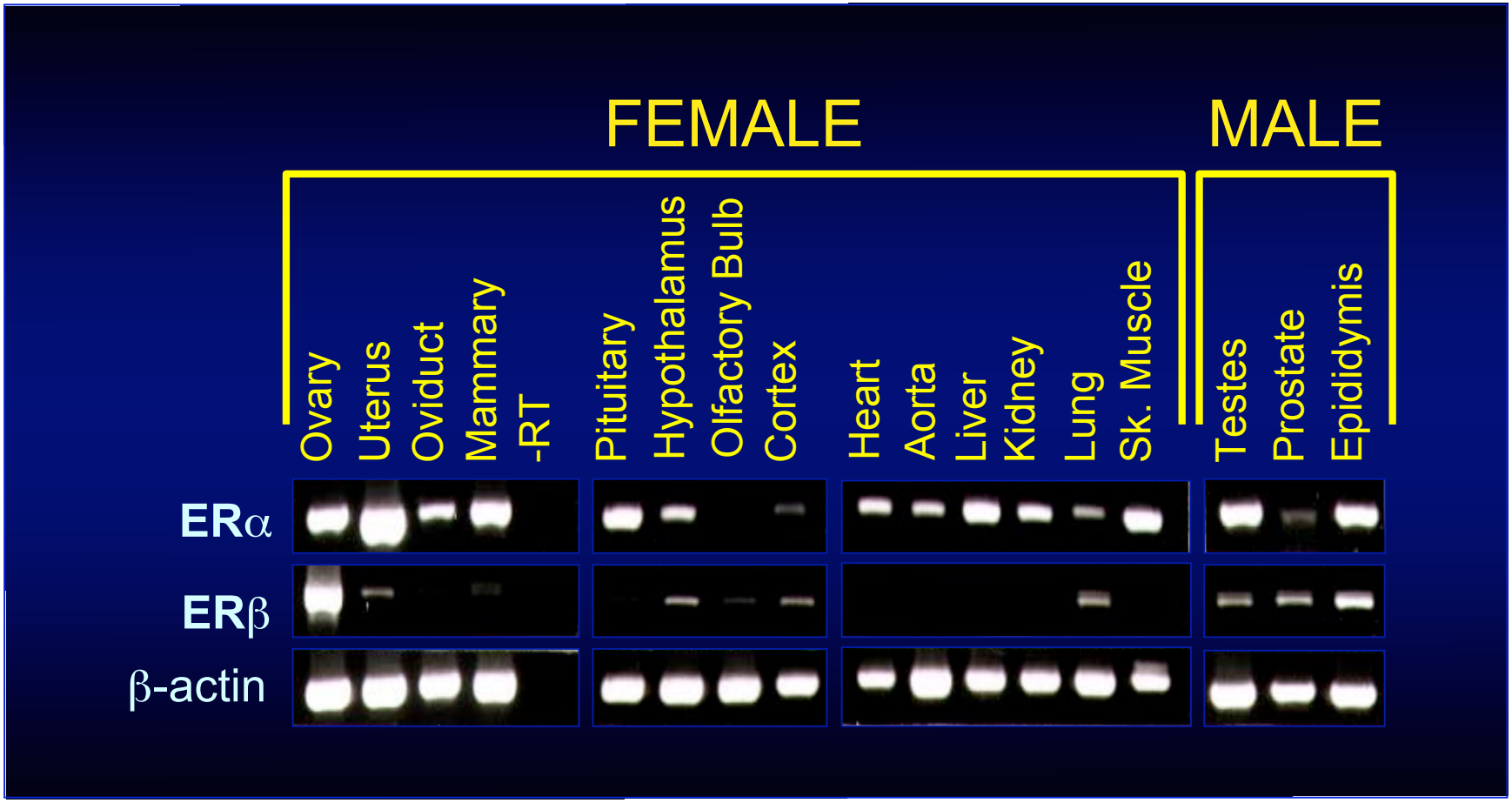
1D PAGE for Vtg

Western for Vtg

Vtg = vitellogenin

RNA/Northern Blot

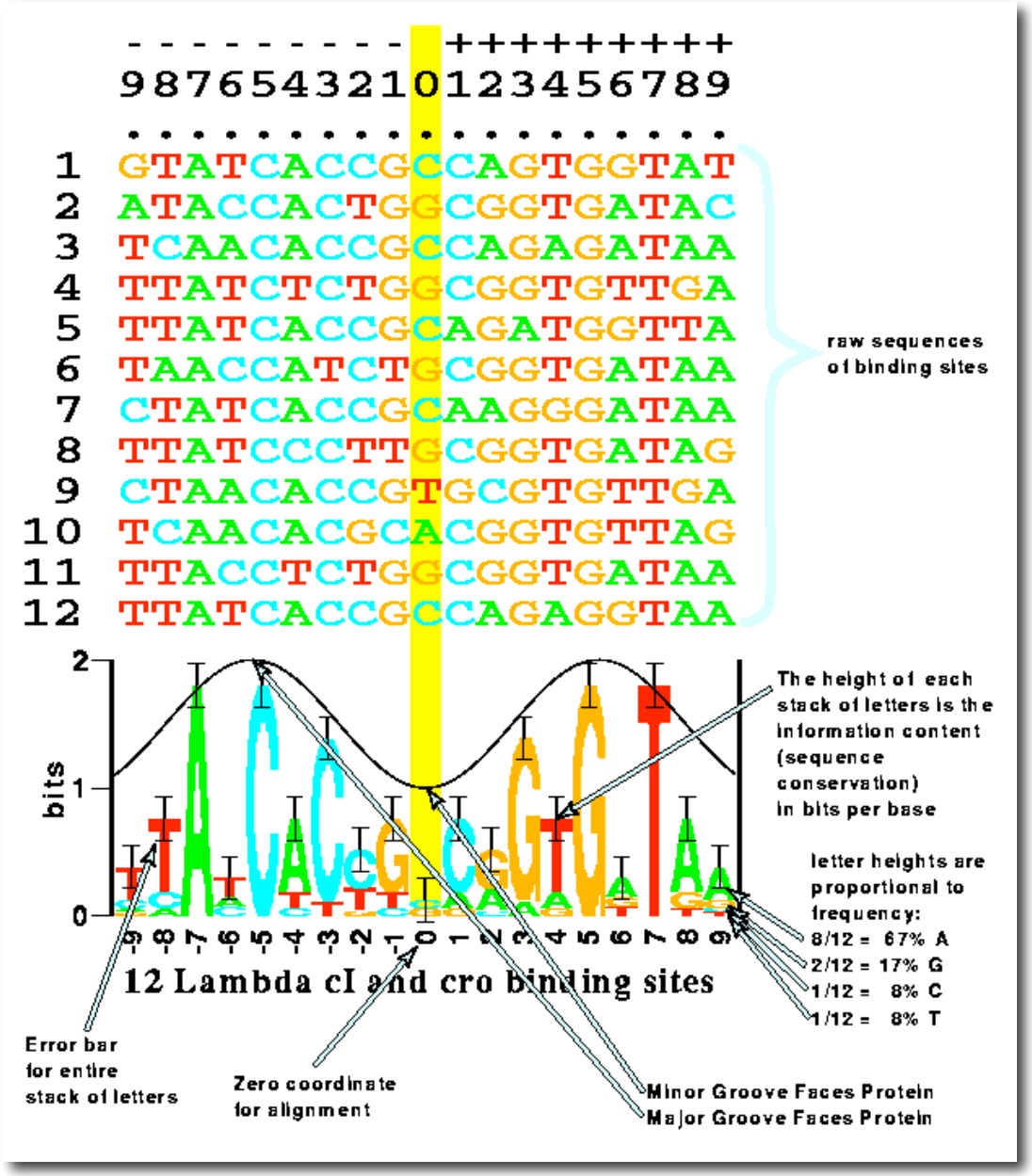
- Electrophoresis of RNA
- Transfer to membrane and probe with complementary radioactive cDNA
- Expose X-ray film/plate



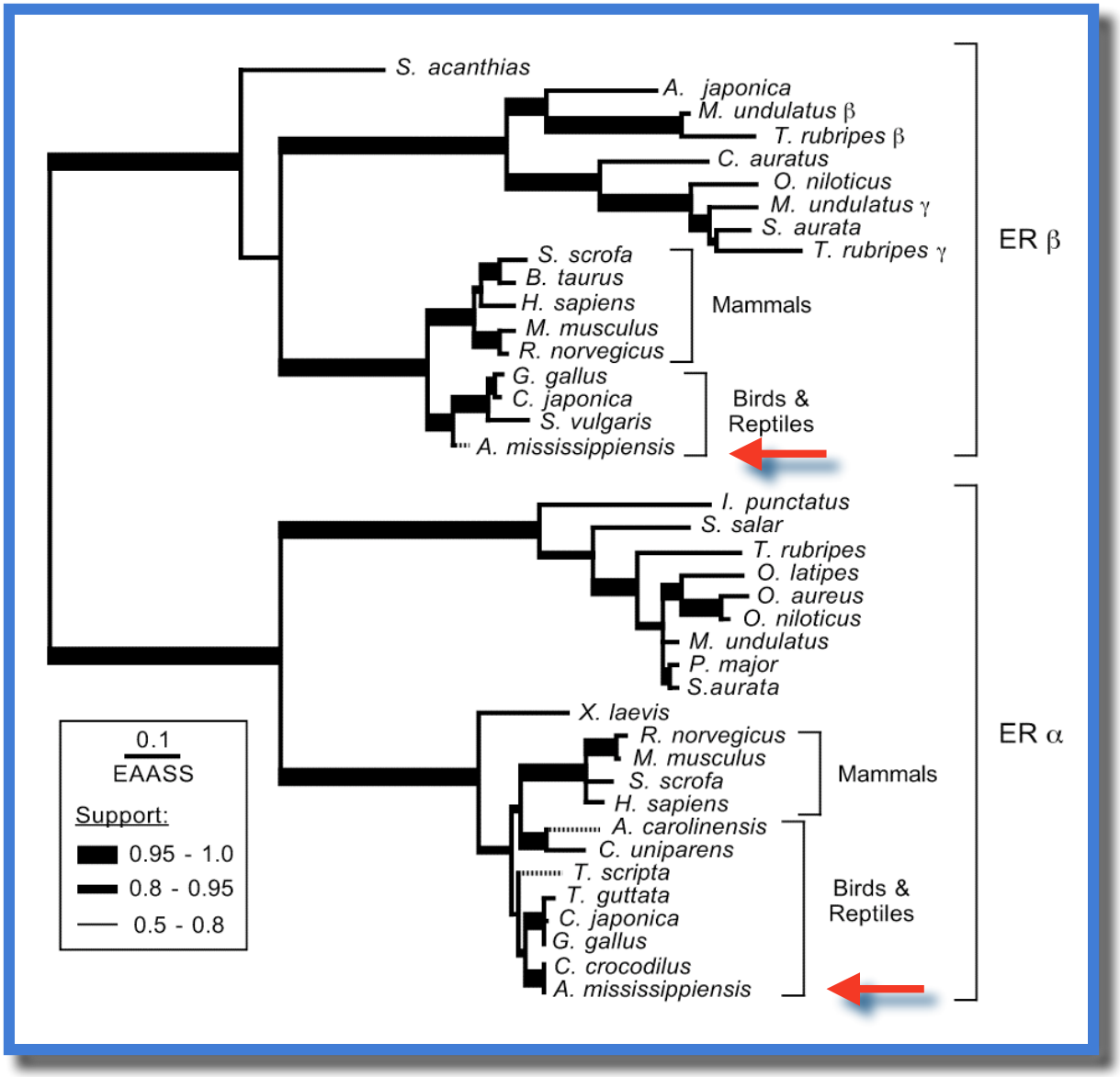
JF Couse and KS Korach (1999) *Endocrine Reviews*. 20:358-417.
JF Couse et al. (1997) *Endocrinology.* 138:4613-4621.

Couse, 1999

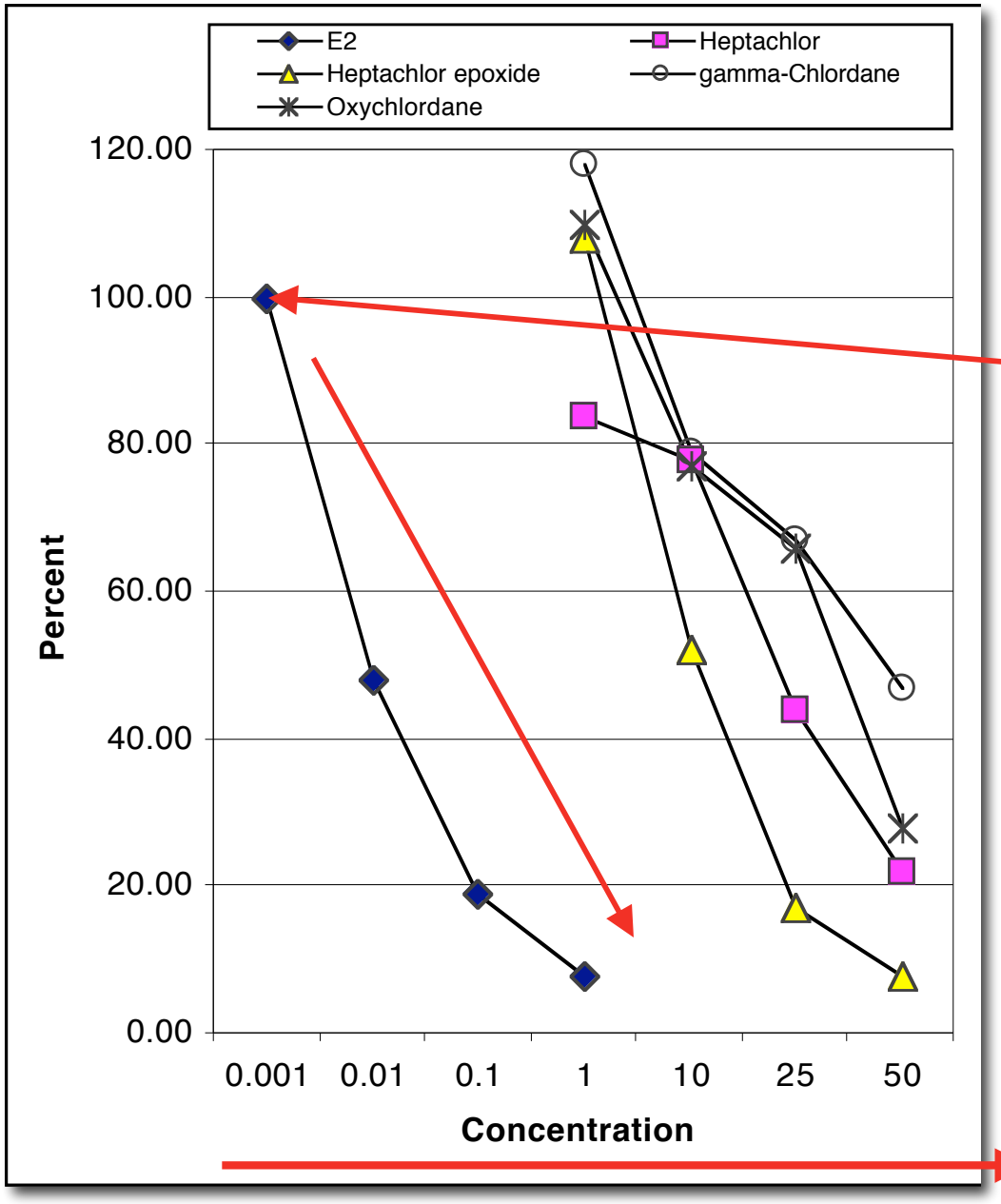
DNA Sequencing



Phylogeny of ER



Katsu, et al., Gen. Comp. Endo. (2003)



ER Binding

Competitive Binding Assay for Receptor Binding

load receptor with radioactive estrogen

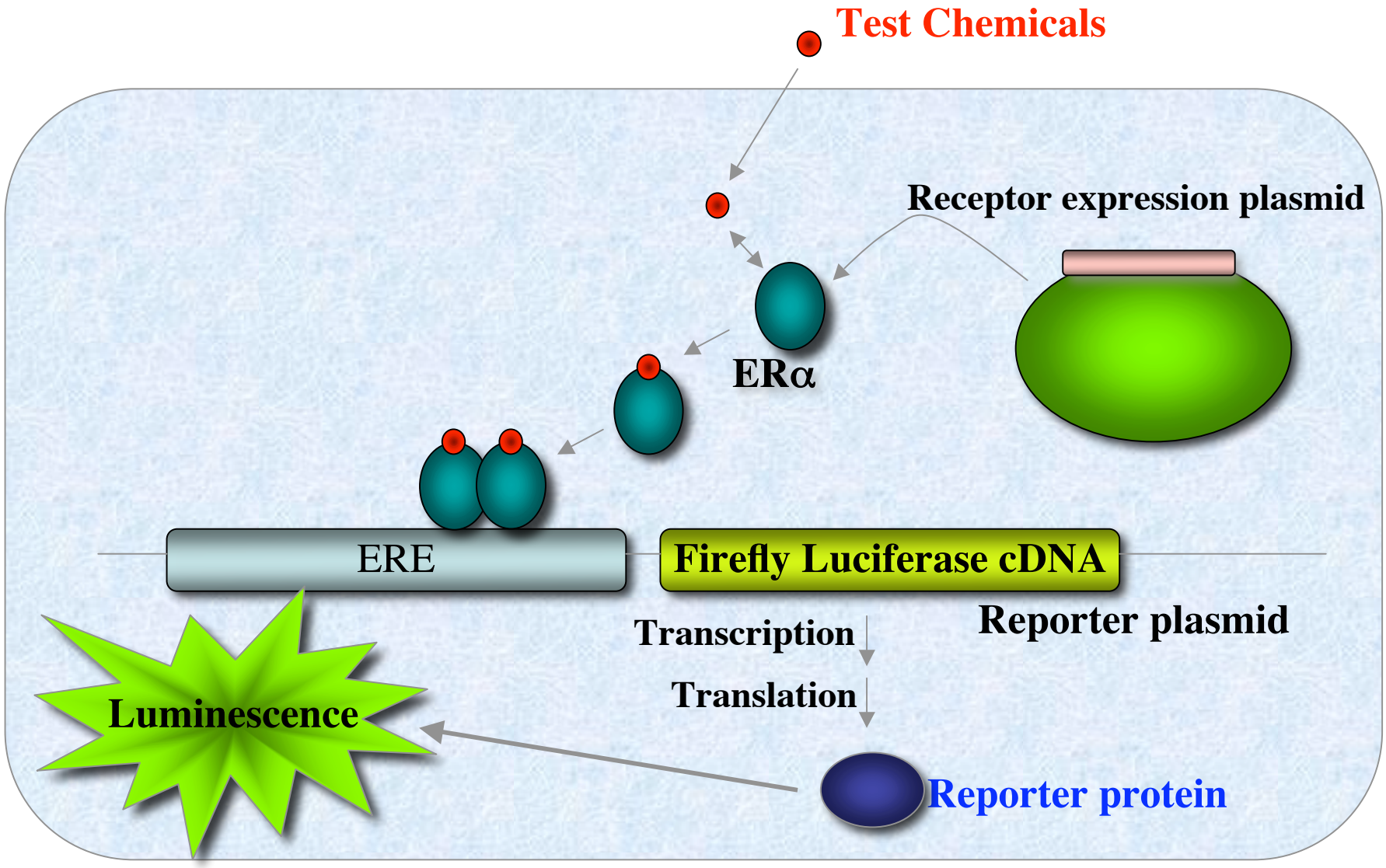
decaying curve

Increase ‘cold’ estrogen

Transfected Cell Line

- Develop a test 'organism'
- Insert a receptor-gene construct if interest for testing
  - Human ER or AR with reporter gene

Reporter Gene Assay using ERE-Luc

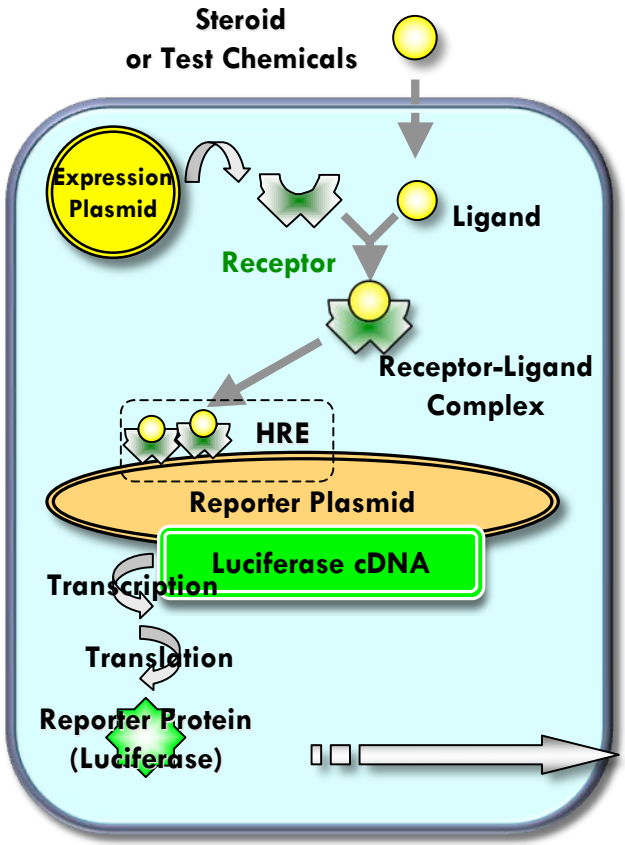


Kohno et al., Integ. Biol. (2007)

Transactivation assays with steroid hormone receptors

Kohno et al. (2008) Integ. Comp. Biology 48:527-534

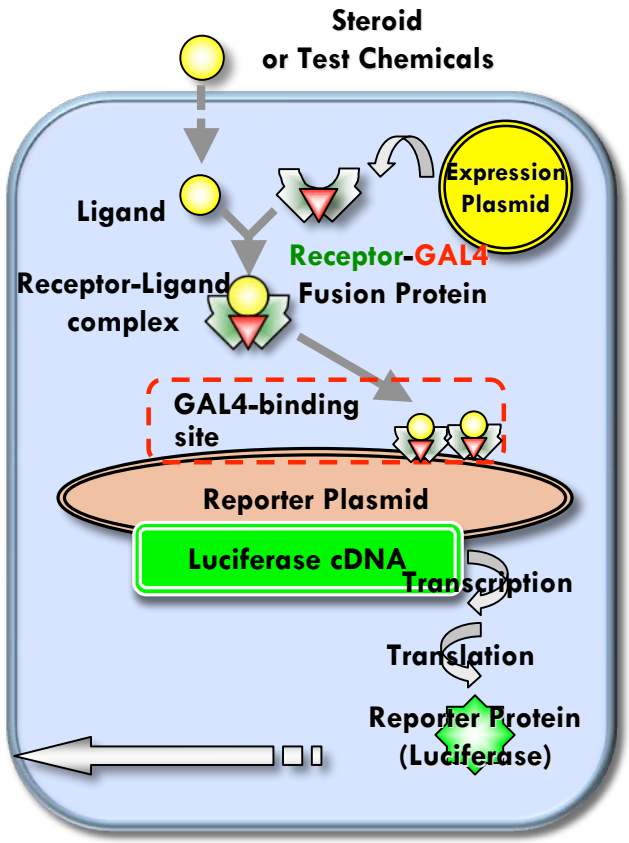
HRE-Luciferase System



estrogen response element-thymidine kinase-luciferase
consensus palindromic ERE

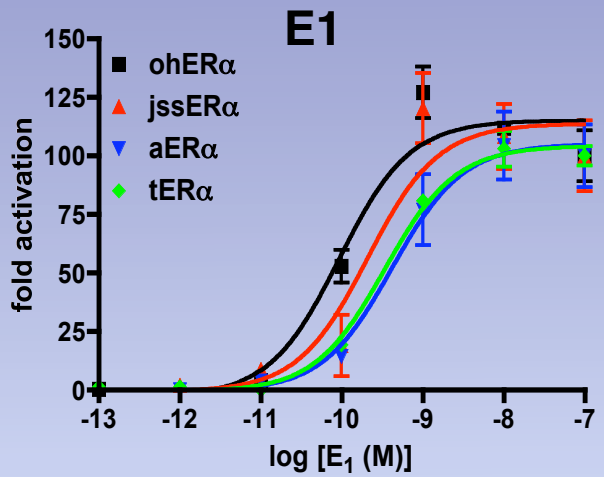
- Induction
  - <10 times
    - vs >100 times
- Promoter specificity
  - HRE: common?
    - vs GAL4: specific
- Recruiting cofactor
  - Both 'unknown'

Modified GAL4 System

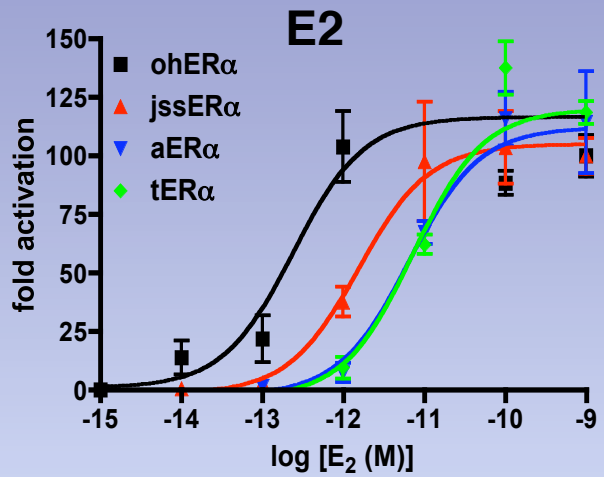


Modified Promega two hybrid system for mammalian cells
CHO-K1 cells

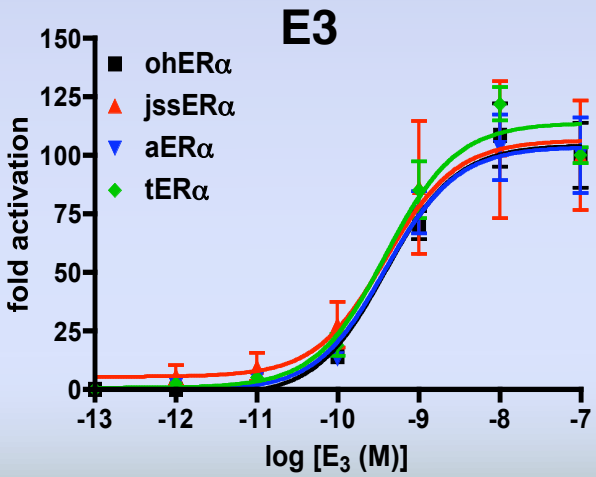
Transactivation of Reptilian ERα Endogenous Estrogens



Viper>Snake>Alligator=Turtle



Viper>Snake>Alligator=Turtle

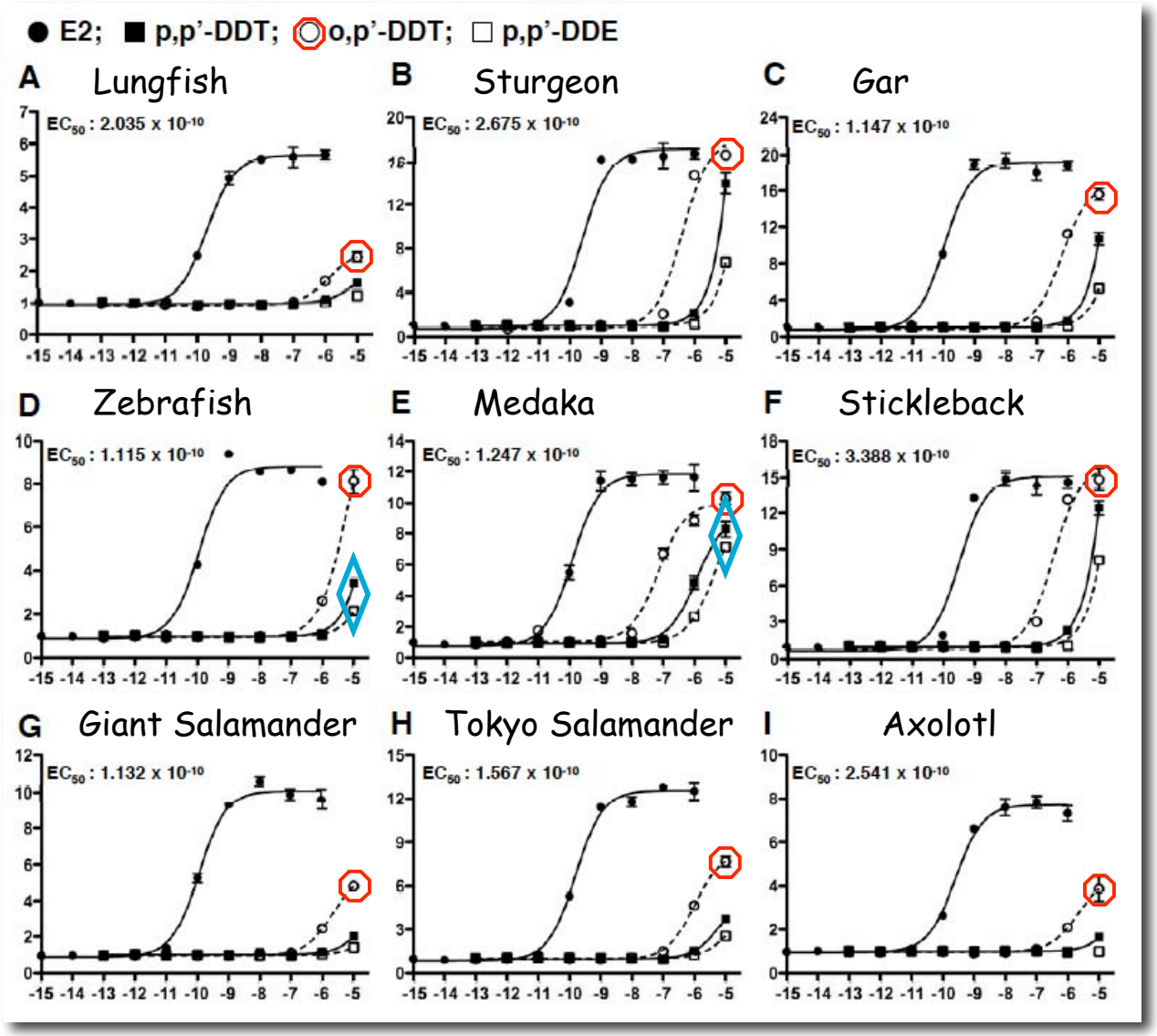


All Equal

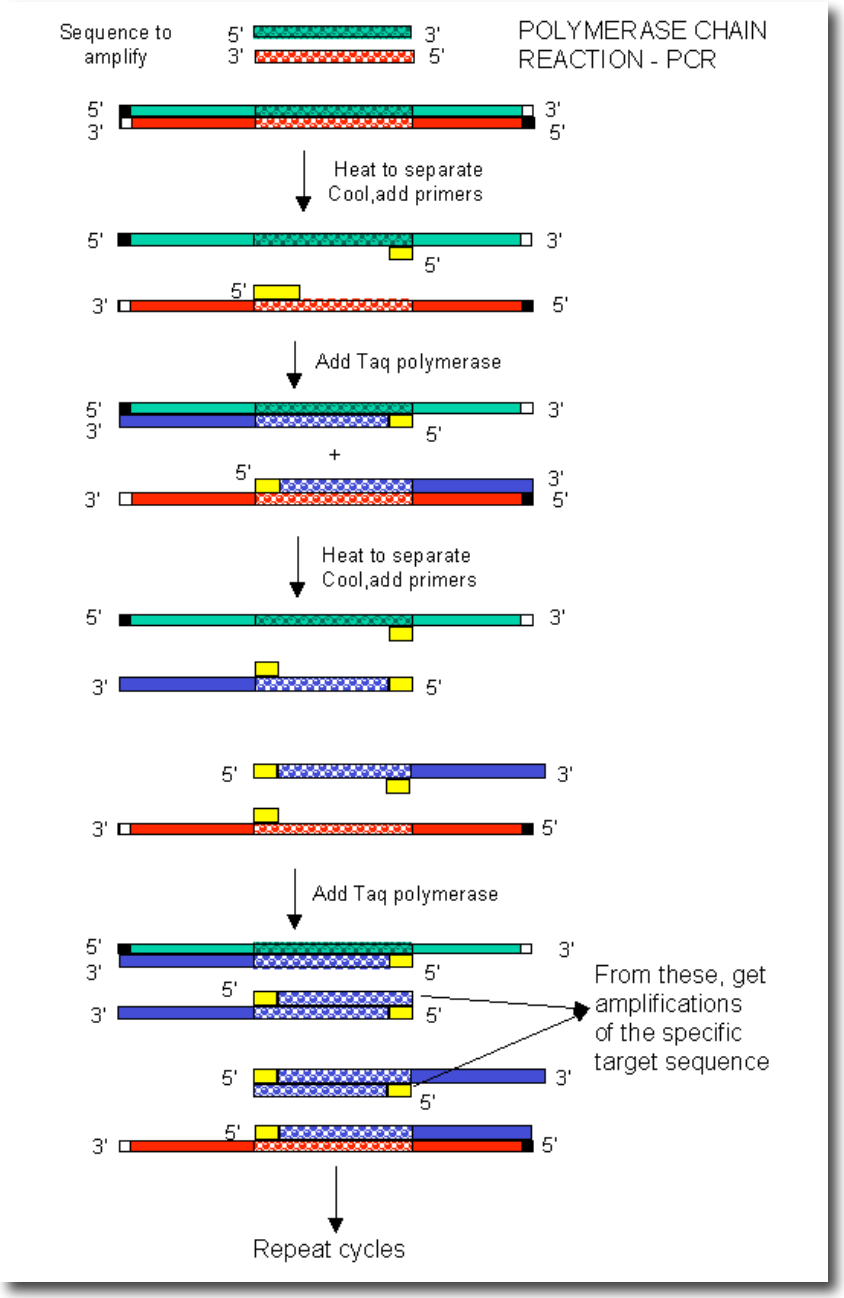
oh: *Trimeresurus flavoviridis* (habu viper)
jss: *Elaphe quadrivirgata* (rat snake)
a: *Alligator mississippiensis* (alligator)
t: *Pseudemys nelsoni* (red-belly turtle)

Katsu et al. unpubl. data

ESR1 (ERα) and Contaminants

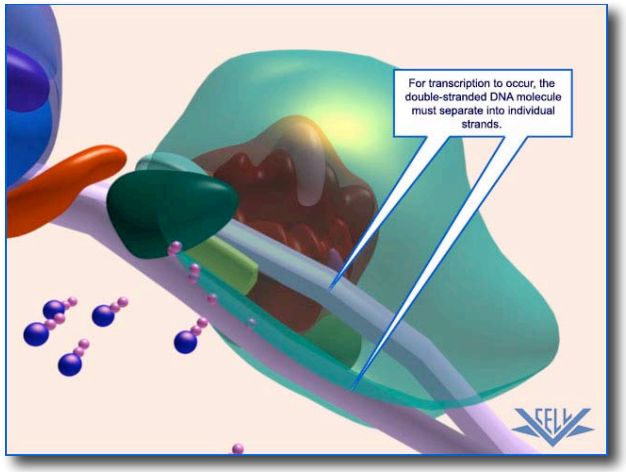


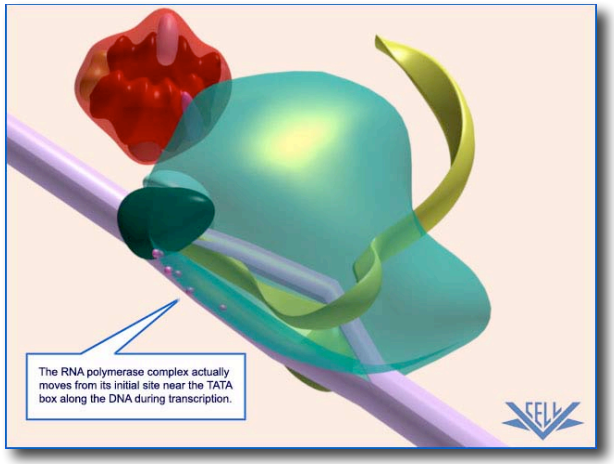
Katsu et al. Mol Cell Endo 2006, 2007; Endocrinology 2008a,b



PCR

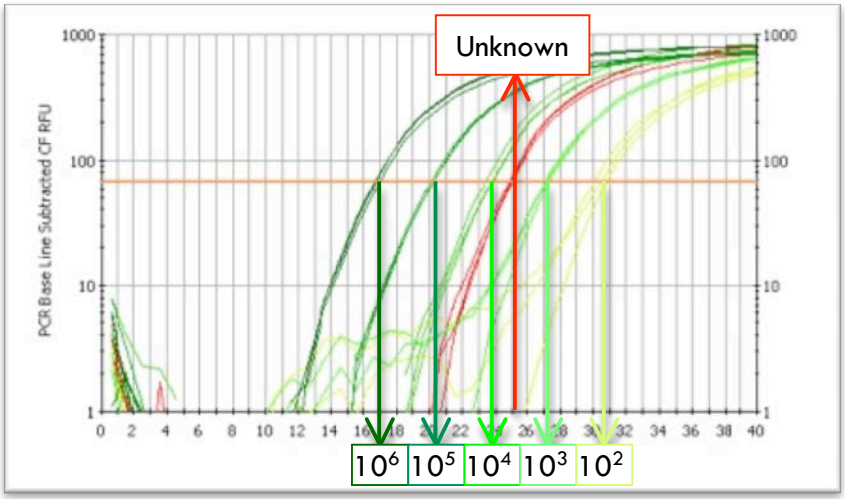
- Use of natural DNA/RNA mechanisms





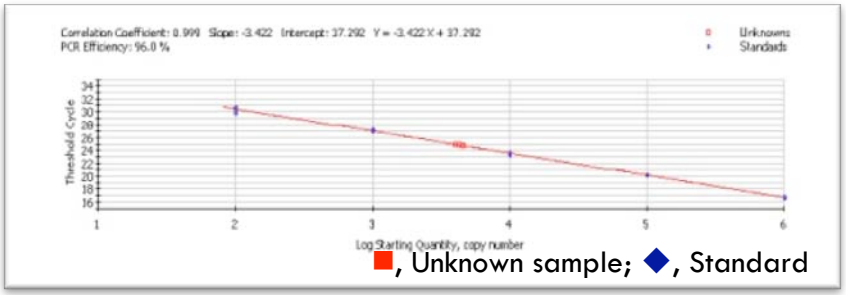
The Outputs Of Q-PCR

Amplification Curve

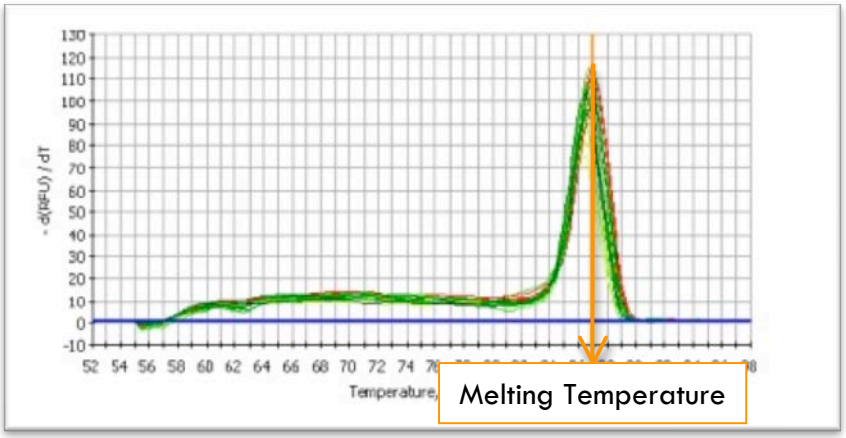


- Unknown Sample
- 10^6 copy/μl
- 10^5 copy/μl
- 10^4 copy/μl
- 10^3 copy/μl
- 10^2 copy/μl

Standard Curve



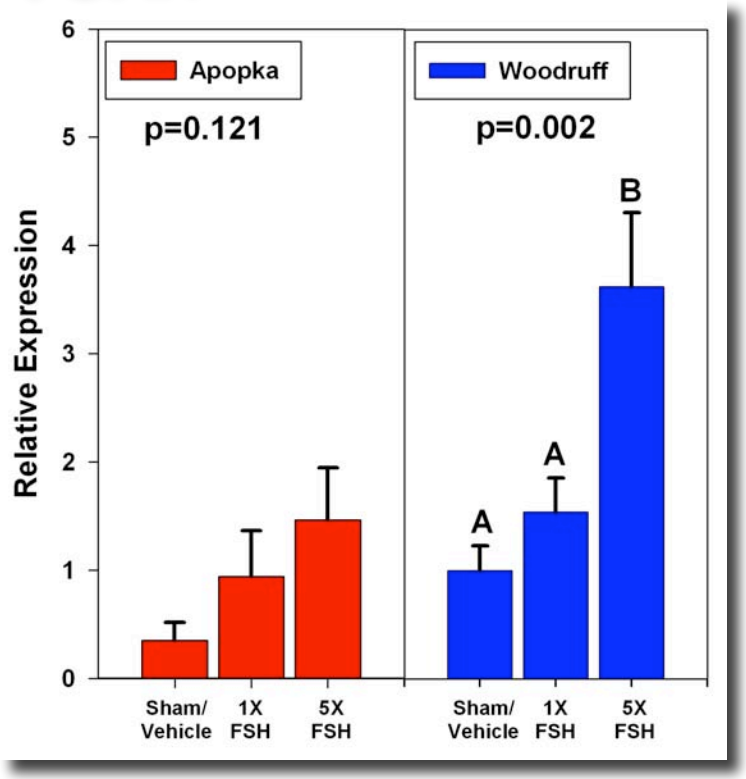
Melting Curve After The Reaction



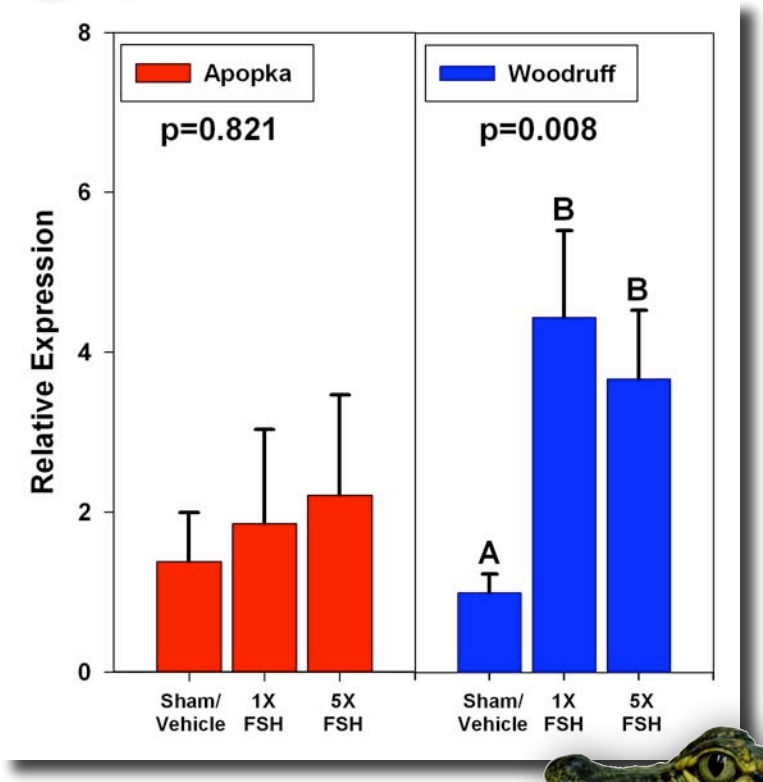
FSH Changes Ovarian Gene Expression

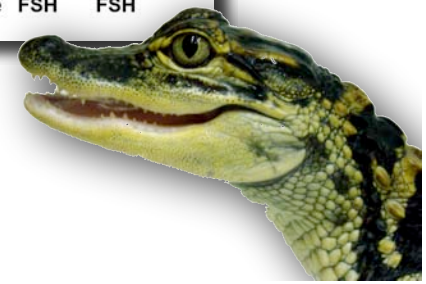
Quantitative Real Time - PCR

FSHR



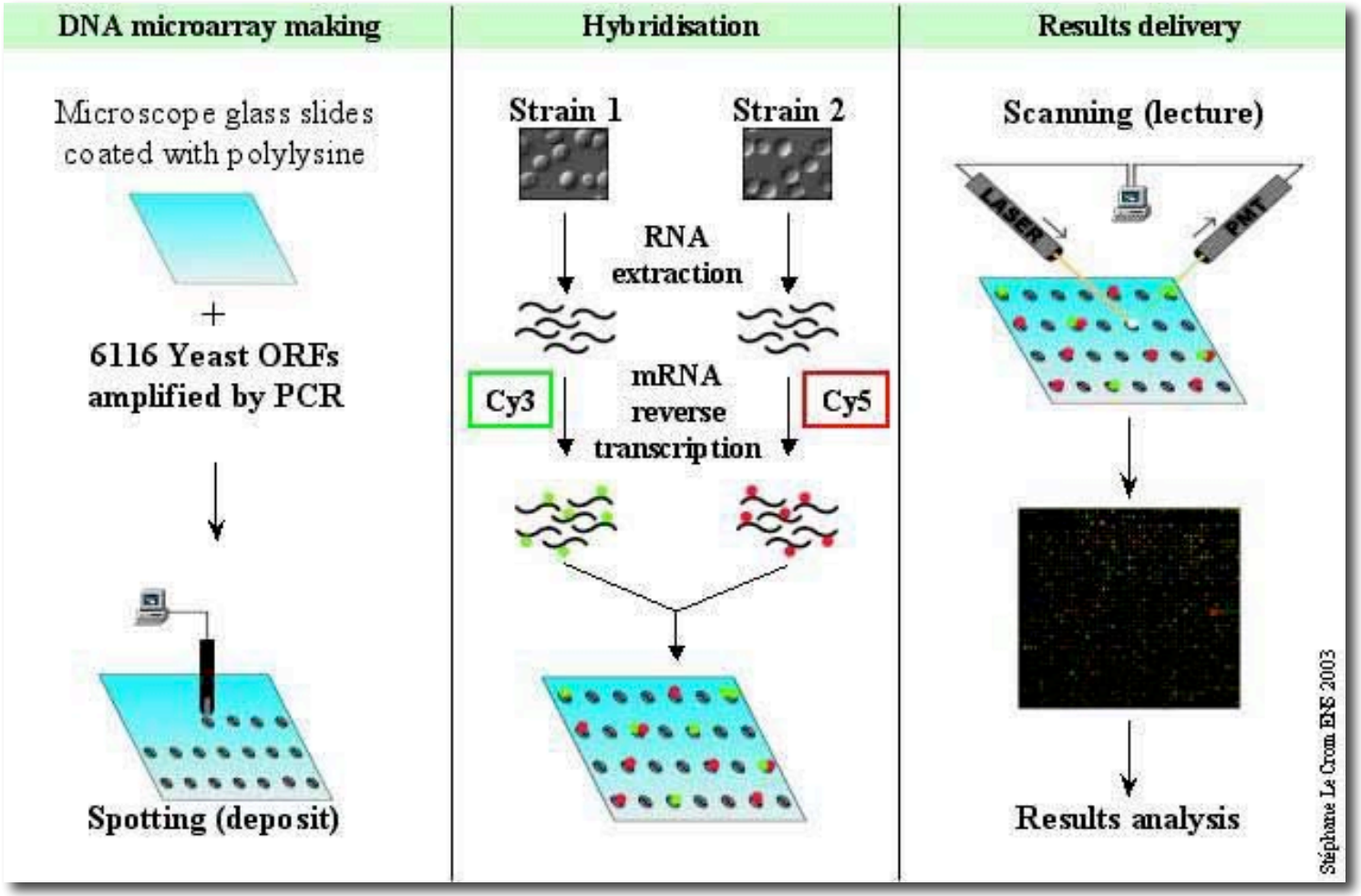
ERα

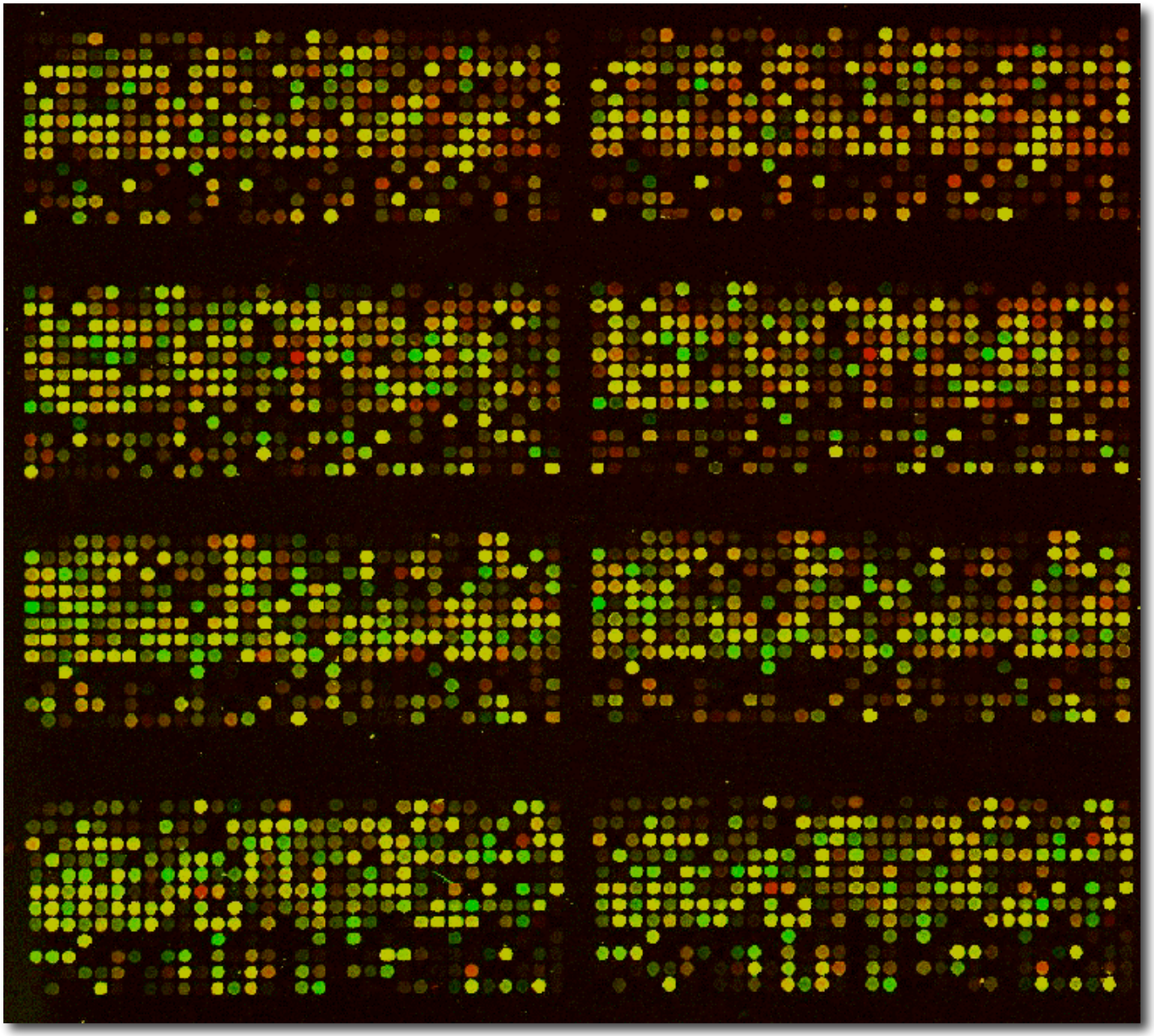




Moore et al. unpubl. data

Microarray


DNA microarray making
Microscope glass slides coated with polylysine
+
6116 Yeast ORFs amplified by PCR
Spotting (deposit)
Hybridisation
Strain 1
Strain 2
RNA extraction
Cy3
Cy5
mRNA reverse transcription
Results delivery
Scanning (lecture)
LASER
PMT
Results analysis
Stéphane Le Crom ENS 2003



Transgenic Animals