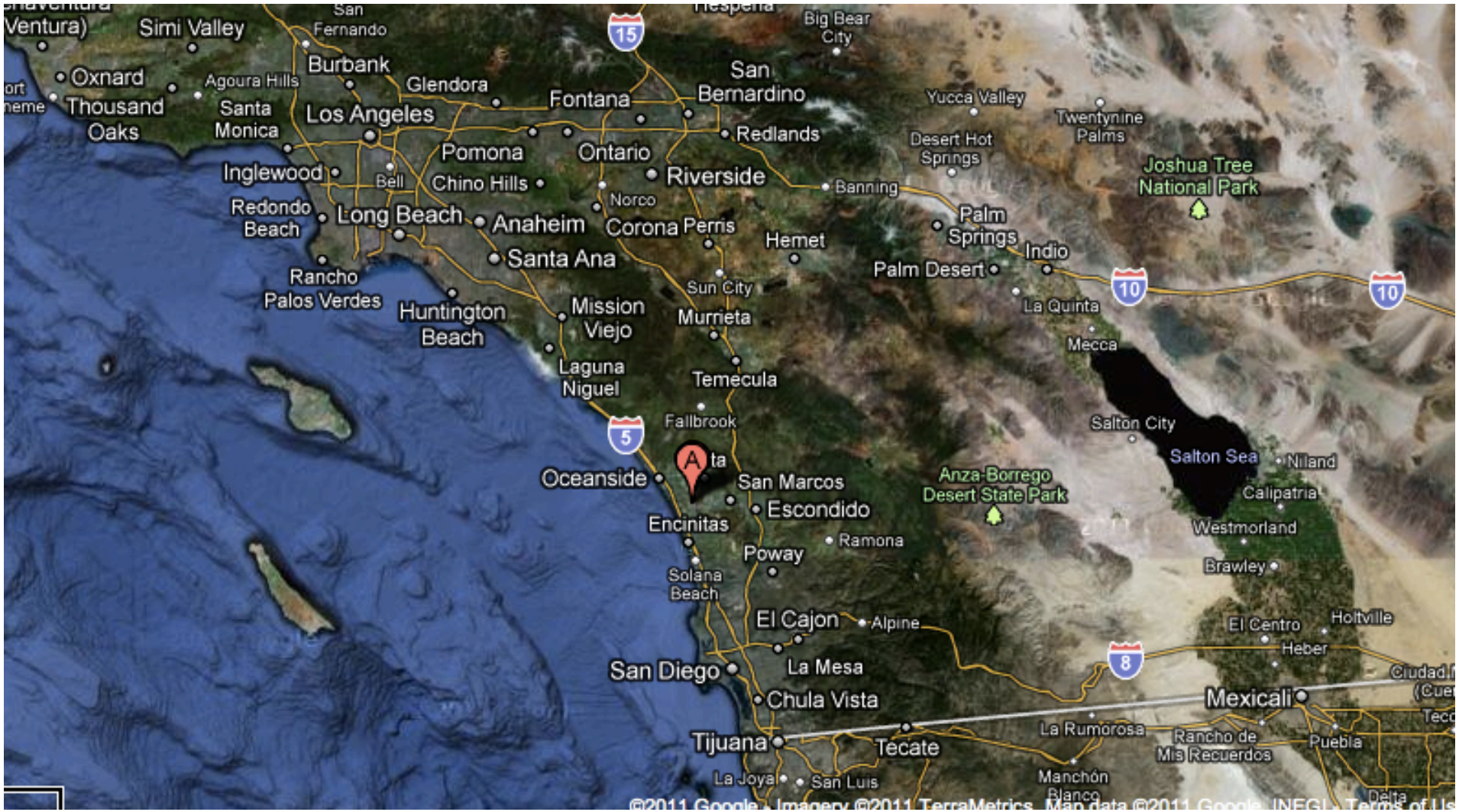
Lab Meeting
Dan
FRIDAY May 20, 2011

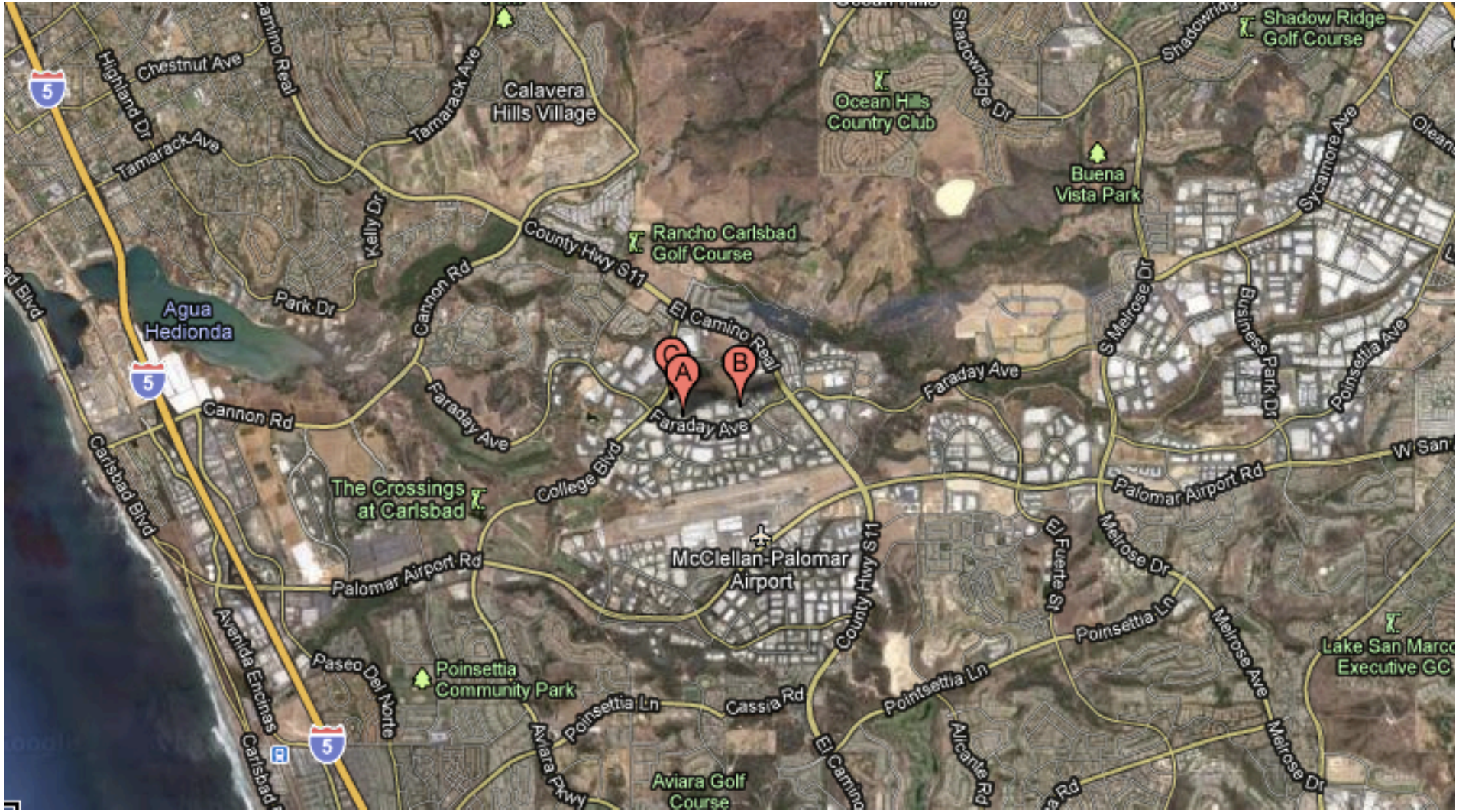
1) ISIS Pharmaceuticals

2) Promoter Paper

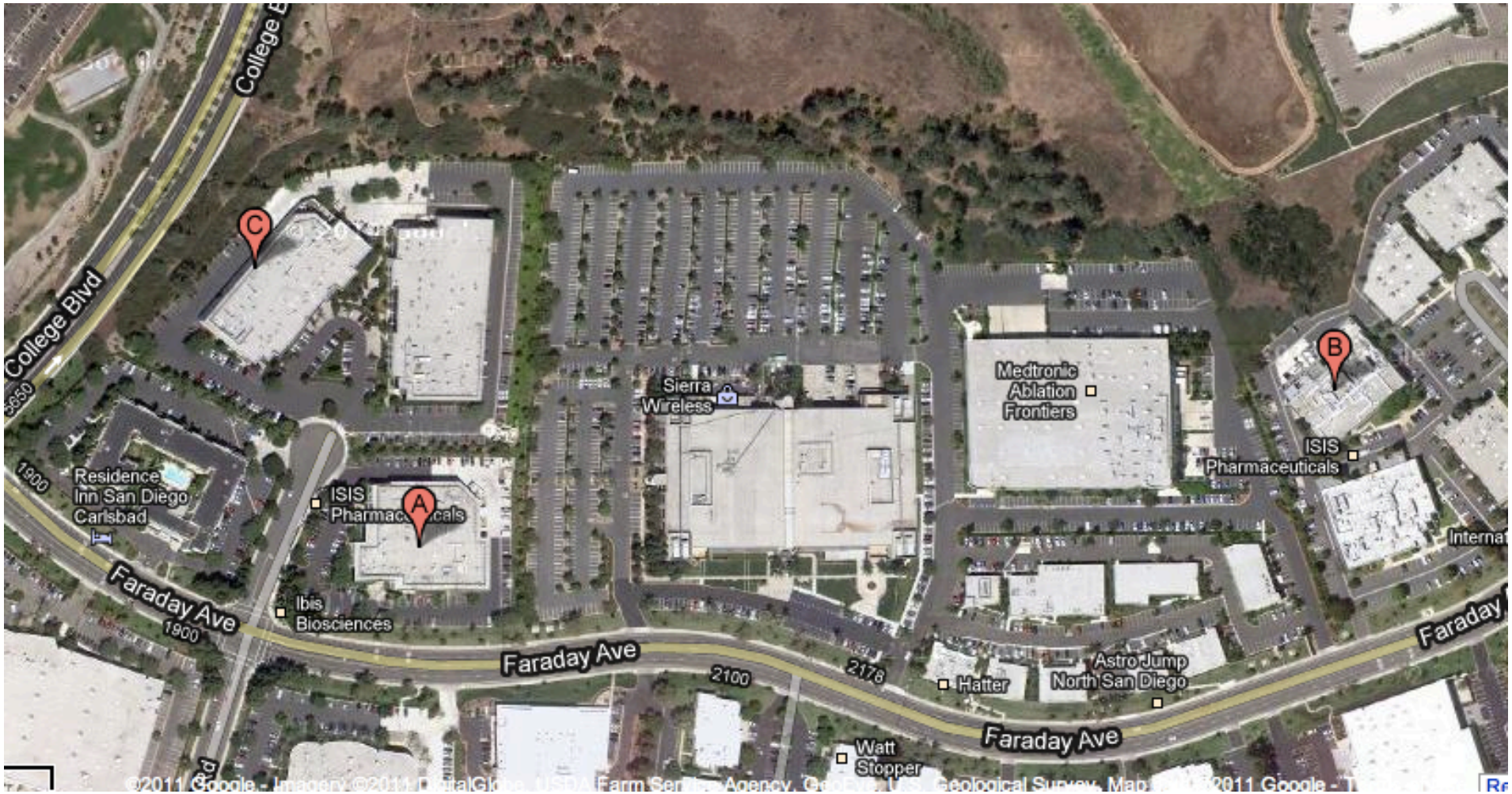
ISIS PHARMACEUTICALS



ISIS PHARMACEUTICALS



ISIS PHARMACEUTICALS



ISIS PHARMACEUTICALS
Future Site

175,000 sq ft.
Targeted completion date:
July 2011

Objectives

- Establish the collaboration

  Meet the players and get everybody on board with the idea

- Learn ICV bolus procedure for mice

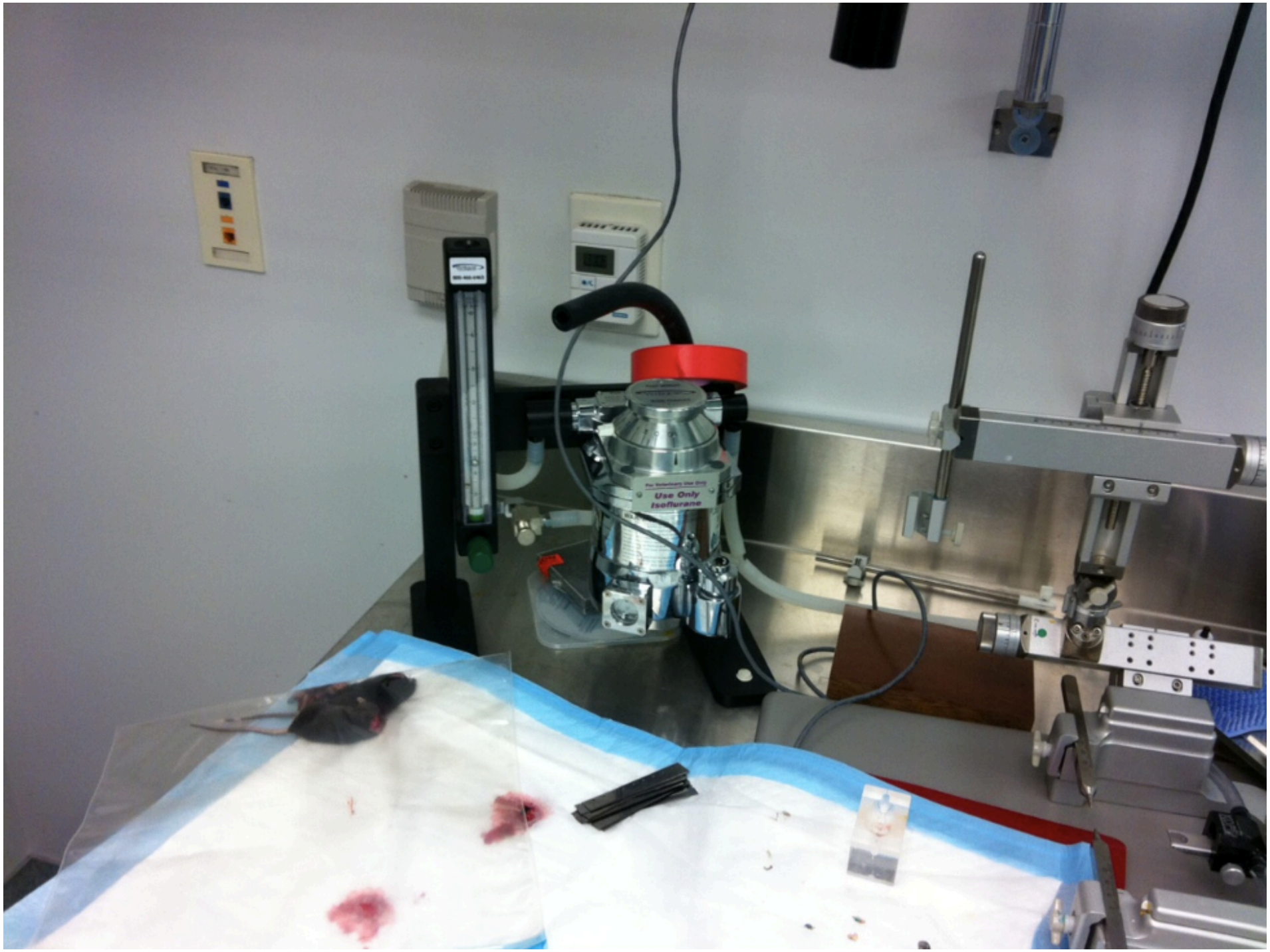
ICV Bolus

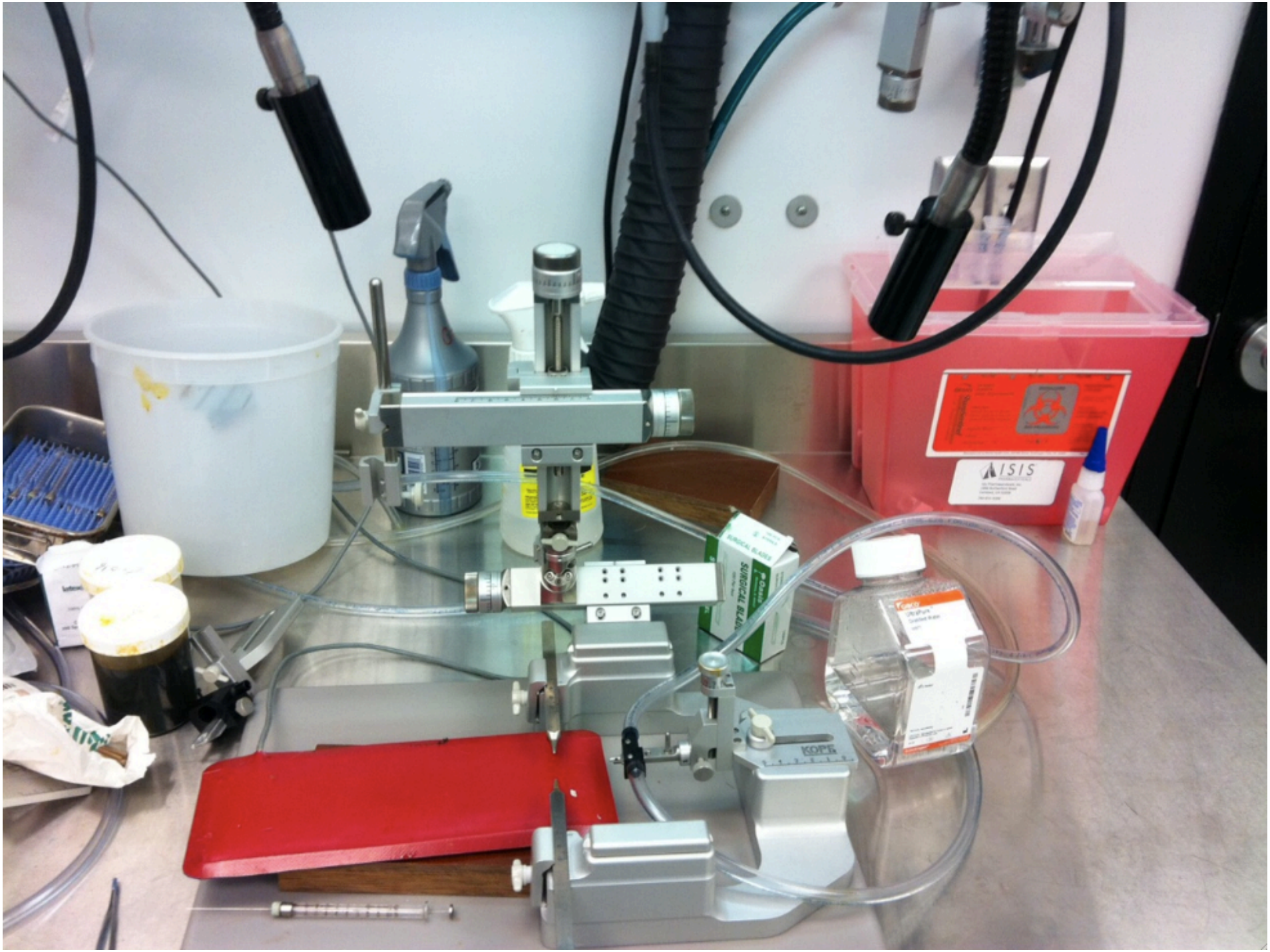
ICV = intracerebroventricular

Bolus = one big slug of drug

IVC up to 10 ul

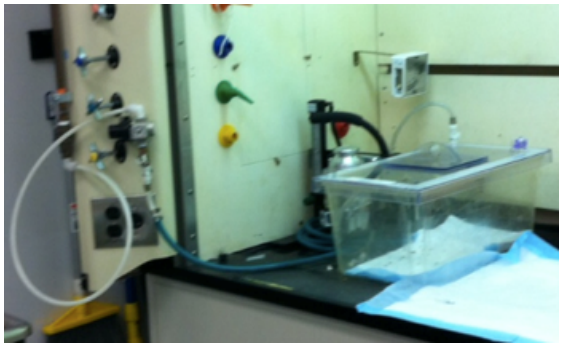

IITC


Use Only
Isoflurane


ISIS
KOPF

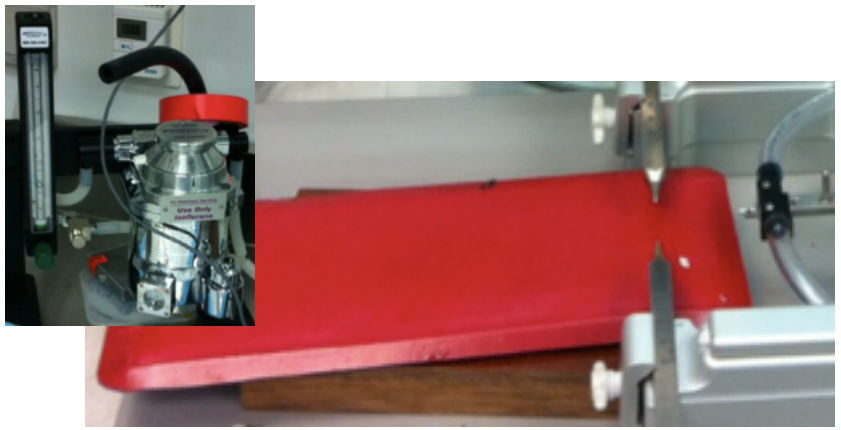
Gas Anesthesia

Induction Chamber

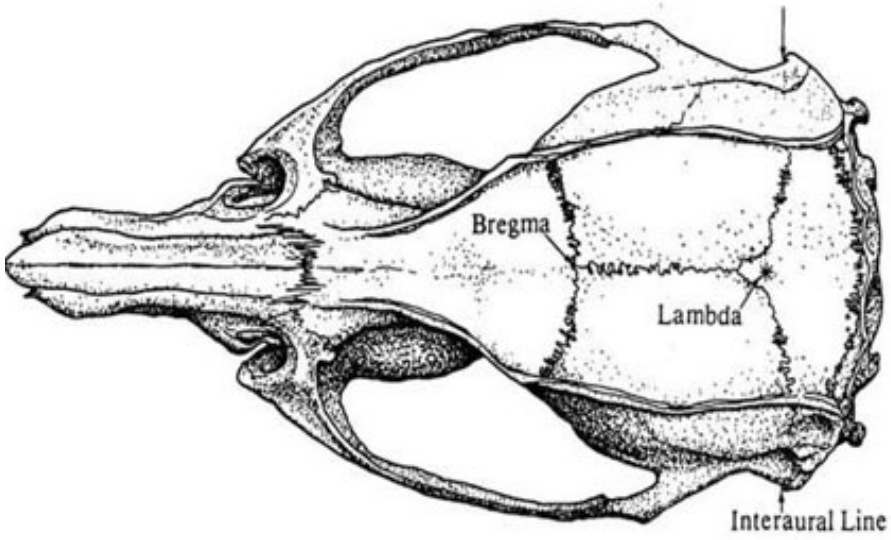


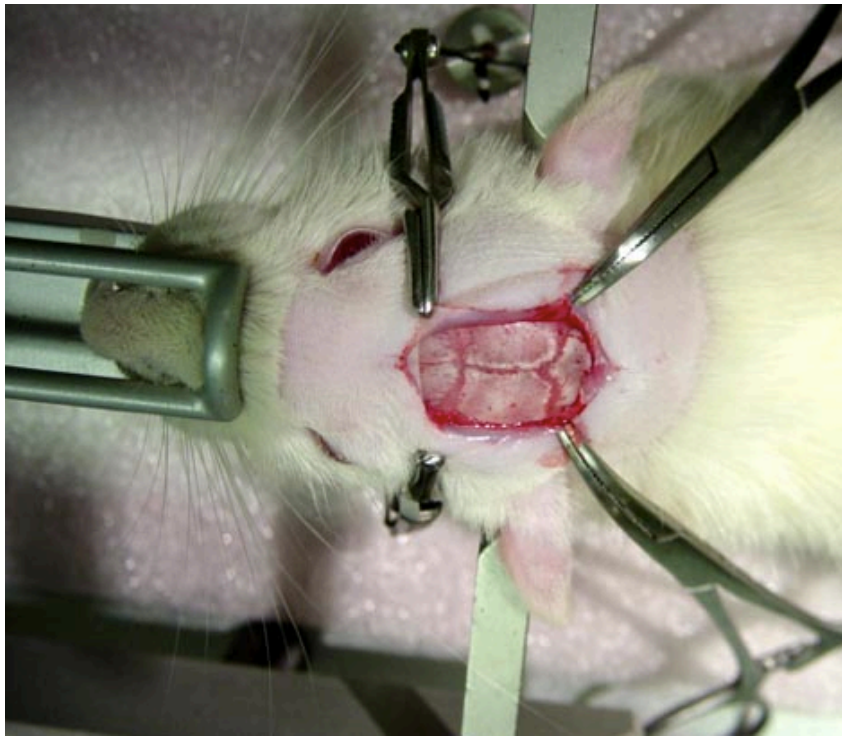
- Air line connected set to 50 psi
- That line connects to a flow regulator set to 3L/min
- The isoflurane gas regulator is set to 3%
- The chamber has the exit vent sealed off.
- After the mouse has been in for about 30s turn down the flow rate to 1L/min.
- Mouse can stay there for a while (5 min), but if too long at 3% will die.

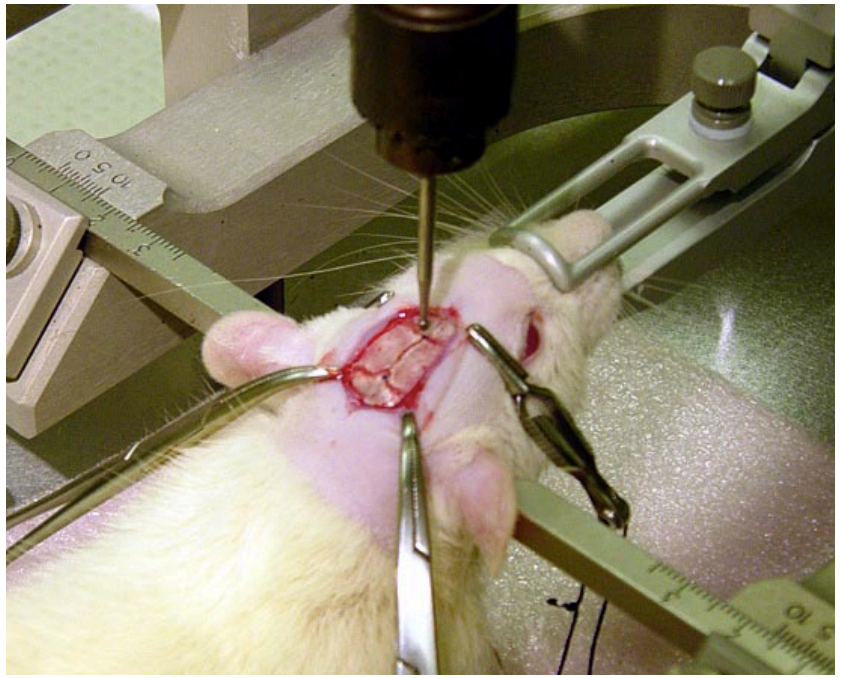
At the Stereotax

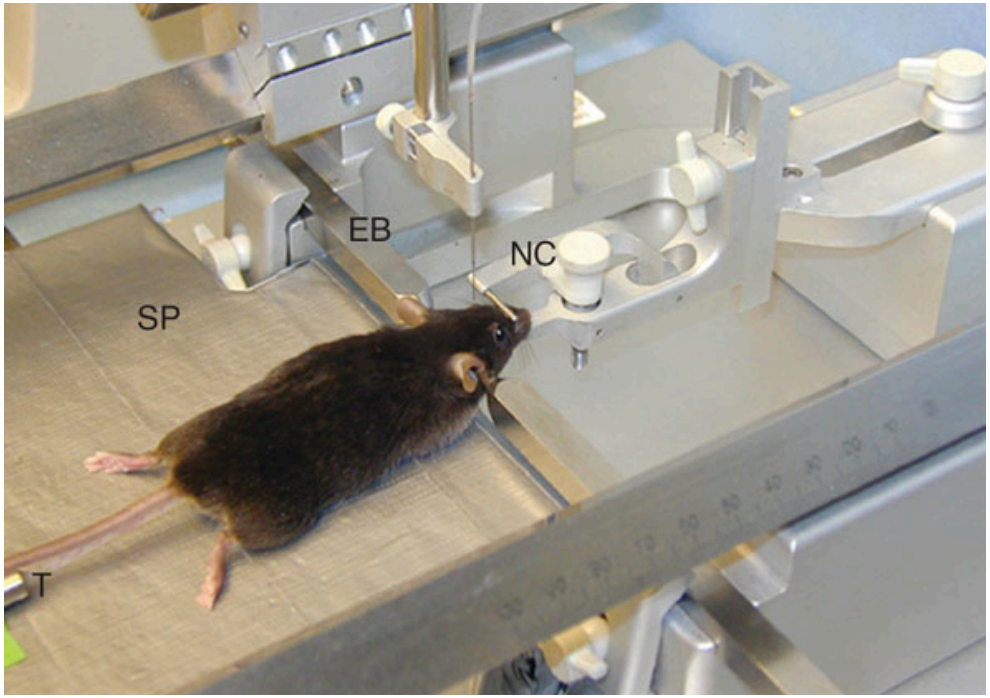


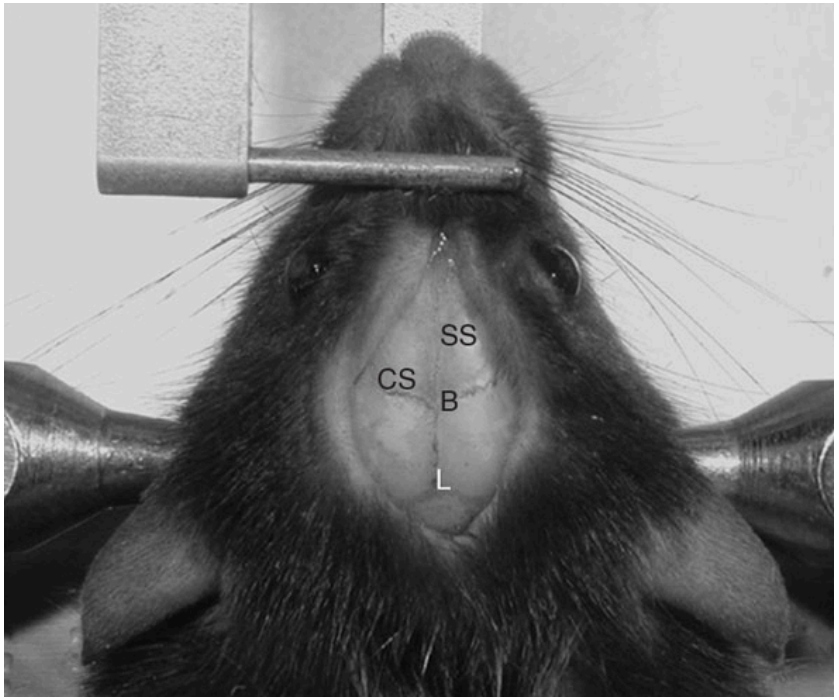
- Oxygen supply line connects to regulator
- Flow regulator set to 0.5L/min/instrument
- The isoflurane gas regulator is set to 2%
- Mouse can stay there longer because of the heat pad (anesthesia causes hypothermia).


Bregma
Lambda
Interaural Line









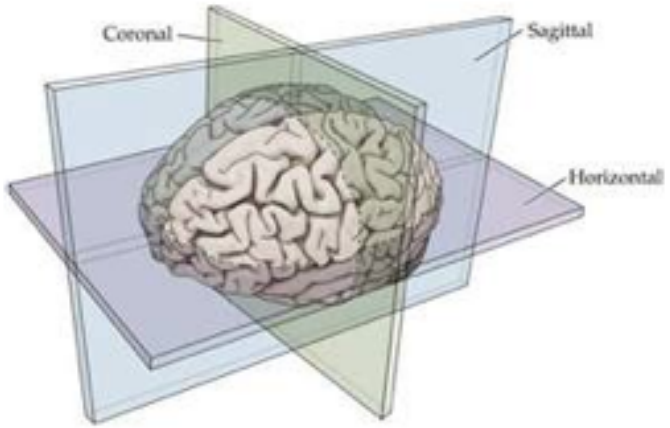
Nature Protocols **3**, 122 - 128 (2008)

Order of Stereotaxic Coordinates : AP-ML-DV

Anterior Posterior (AP) (coronal plane)

Medial Lateral (ML) (sagital plane)

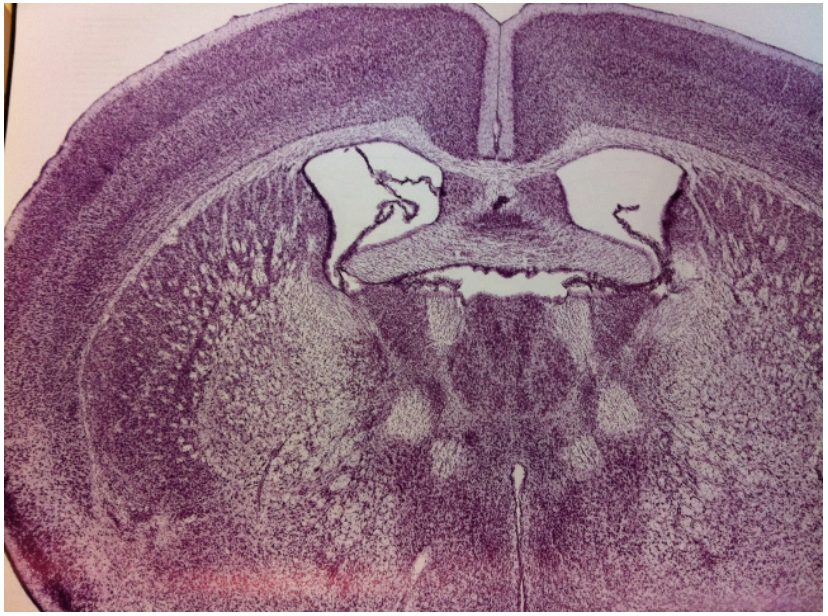
Dorsal Ventral (DV) (axial plane)

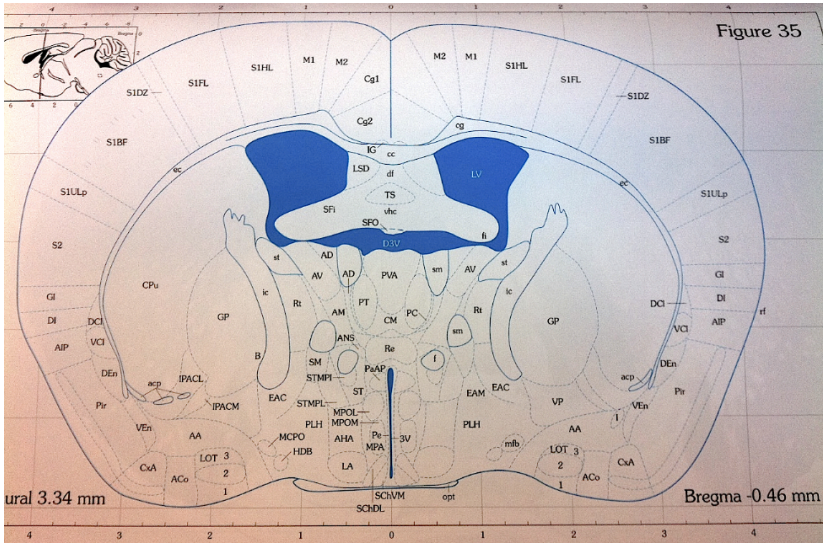


Franklin and Paxinos

The Mouse Brain in Stereotaxic Coordinates

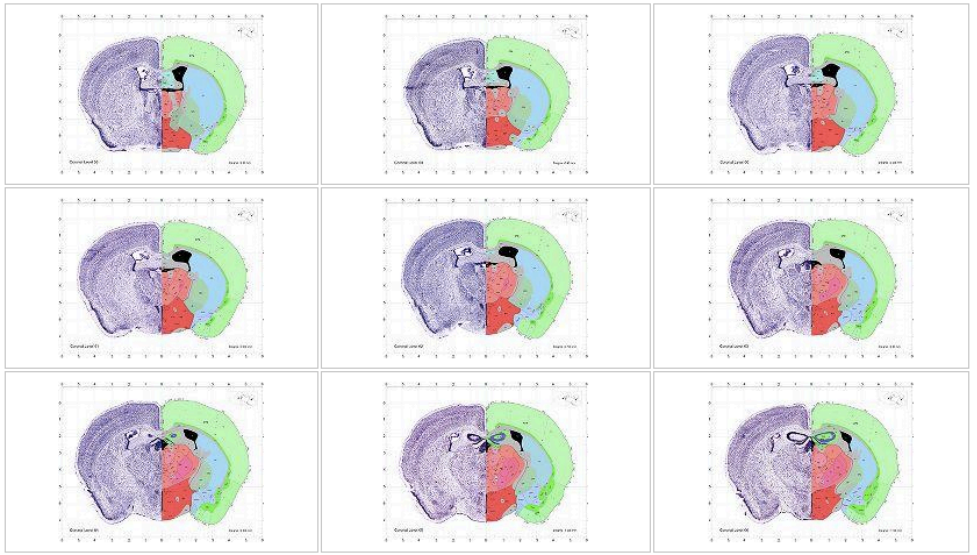
-0.46 x 1 x 2.5

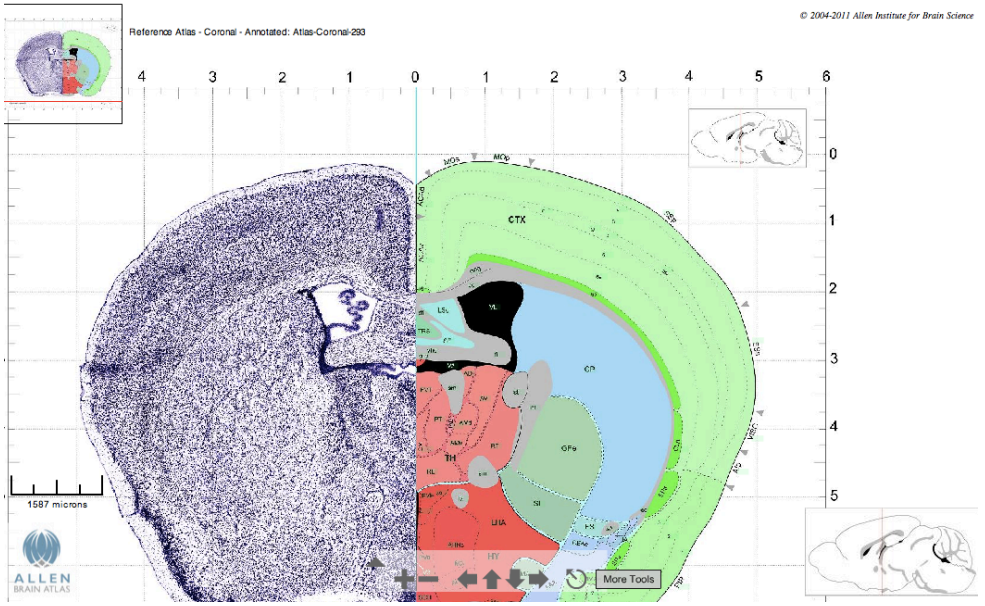




Allen Atlas

-0.48 x 1 x 2.5





Insert Hamilton syringe needle

Inject 1 ul per second counting Mississippis

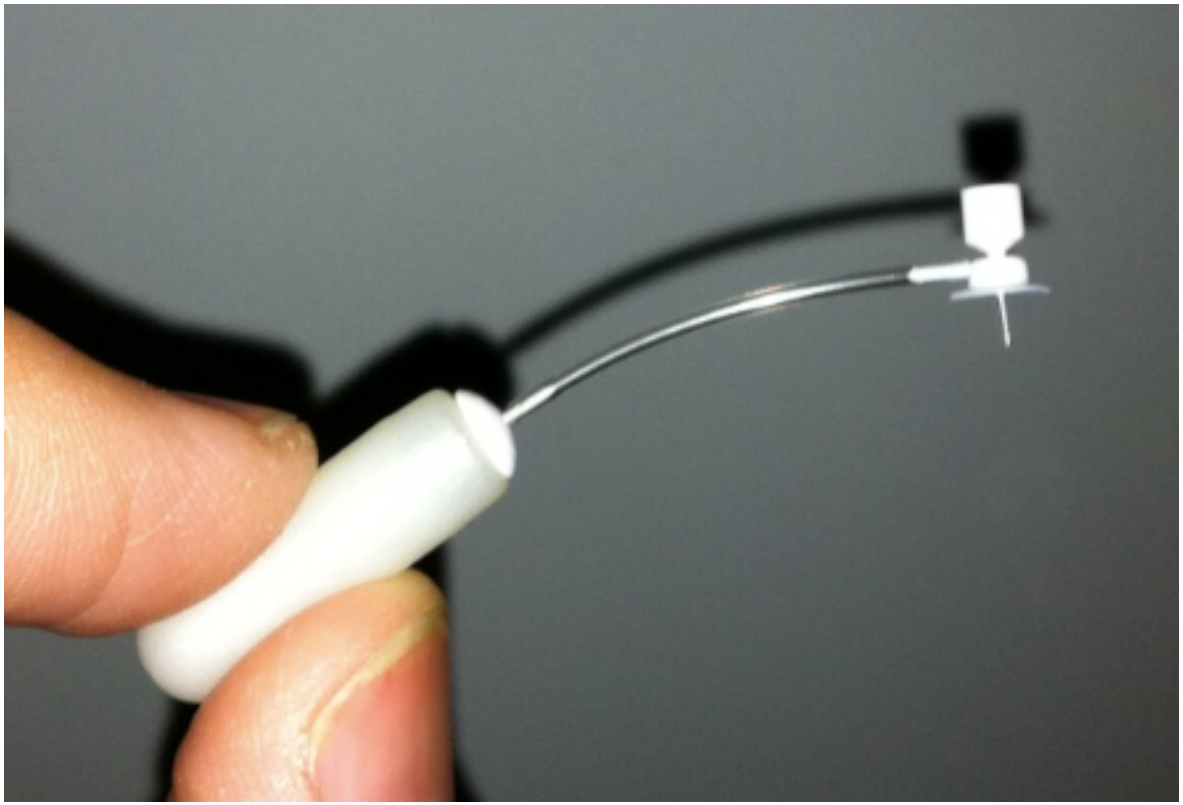
Remove needle, wait 4 min, suture

Finish up with a standard surgeon's knot

Olsen Hegar Needle Holder 4.5" (with scissors) Tungsten Carbide Economy

Sutures: 5-0 Ethilon sold by Ethicon
Non-absorbable. Can use nylon…
Medvet International sells cheaper substitute
shopmedvet.com

Cannula for mice



ASO delivery in humans

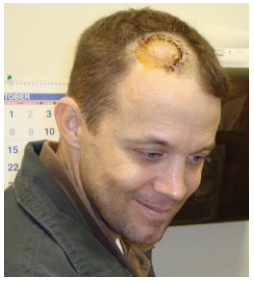
- Intrathecal
  - Intracerebroventricular
    - Bolus
    - Ommya Reservoir
  - Lumbar Puncture
    - Bolus
    - Intrathecal Pump

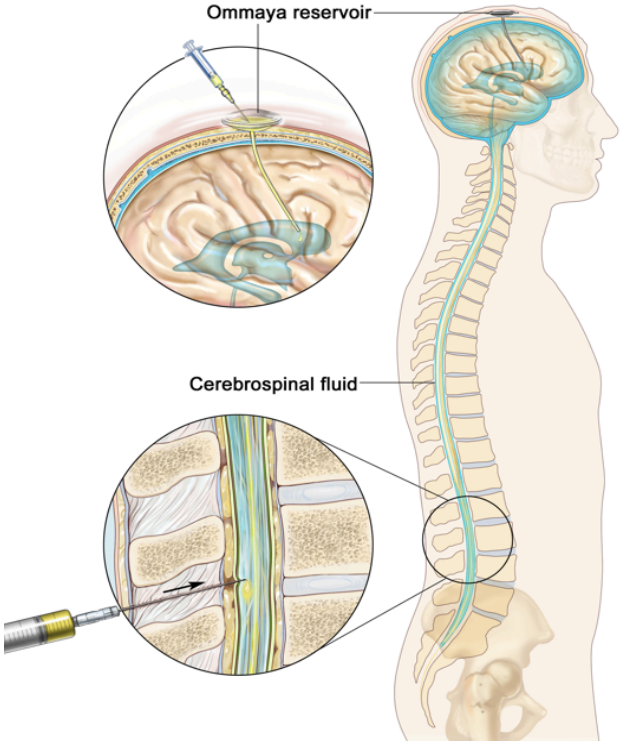
Discussion on different approaches:

A.G. de Boer and P.J. Gaillard. Drug Targeting to the Brain. Annual Review of Pharmacology and Toxicology 47: 323-355 (2007)

Intrathecal Drug Delivery

Ommaya Reservoir





Cisterna magna injection ?

Intrathecal Bolus Injection

Intrathecal Pump

Safety, Tolerability, and Activity Study of ISIS SOD1Rx to Treat Familial Amyotrophic Lateral Sclerosis (ALS) Caused by SOD1 Gene Mutations (SOD-1)

This study is currently recruiting participants.

Verified on September 2010 by Isis Pharmaceuticals

First Received on December 30, 2009. Last Updated on March 22, 2011 History of Changes

Sponsor:	**Isis** Pharmaceuticals
Collaborators:	Muscular Dystrophy Association **ALS** Association
Information provided by:	Isis Pharmaceuticals
ClinicalTrials.gov Identifier:	NCT01041222

Arms
Arm 1: Experimental 0.15 mg **ISIS** 333611 continuous intrathecal infusion over 12 hours Intervention: Drug: **ISIS** 333611
Arm 2: Experimental 0.5 mg **ISIS** 333611 continuous intrathecal infusion over 12 hours Intervention: Drug: **ISIS** 333611
Arm 3: Experimental 1.5 mg **ISIS** 333611 continuous intrathecal infusion over 12 hours Intervention: Drug: **ISIS** 333611
Arm 4: Experimental 3.0 mg **ISIS** 333611 continuous intrathecal infusion over 12 hours Intervention: Drug: **ISIS** 333611
Placebo (phosphate buffered saline): Placebo Comparator Intervention: Drug: **ISIS** 333611

Upcoming trials will be for SMA

Likely will see a trial on HD

ASO cellular entry

ASO distribution is thought to be due to protein binding and facilitated by endocytosis.

The mechanism is not fully known by ISIS

Different chemistries alter ASO effectiveness and is in part thought to be due to cellular entry differences caused by different protein binding properties.

ASO chemistry

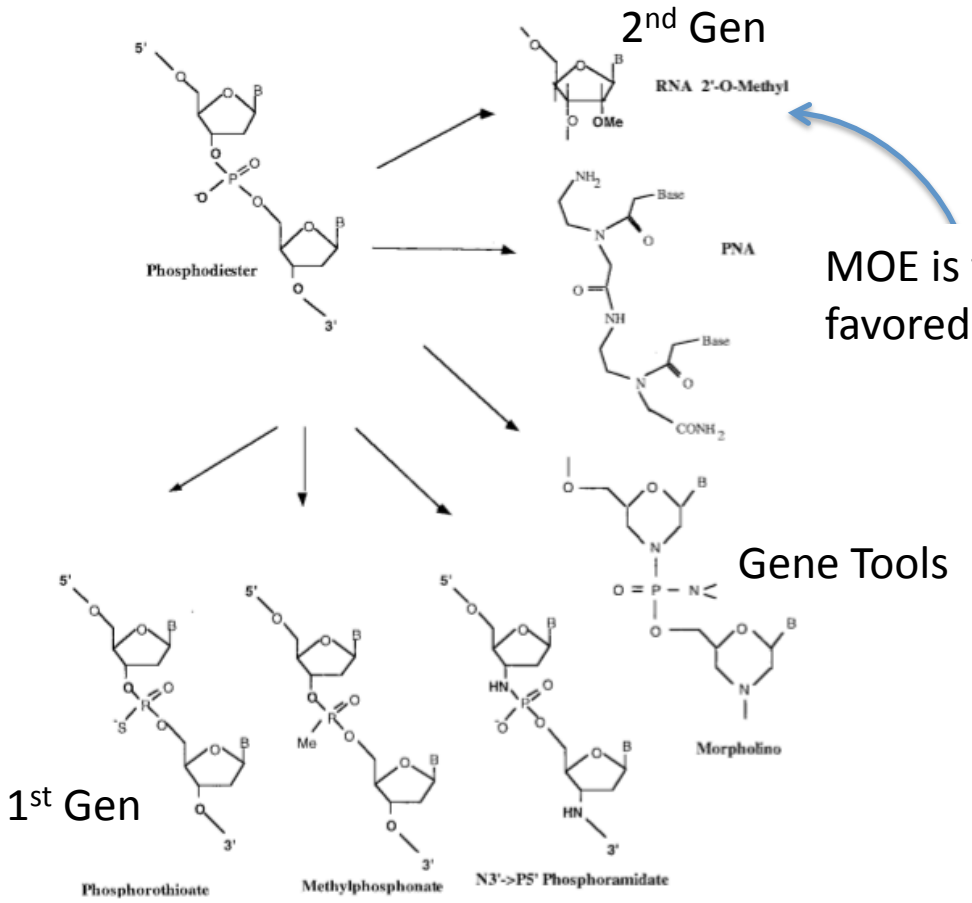
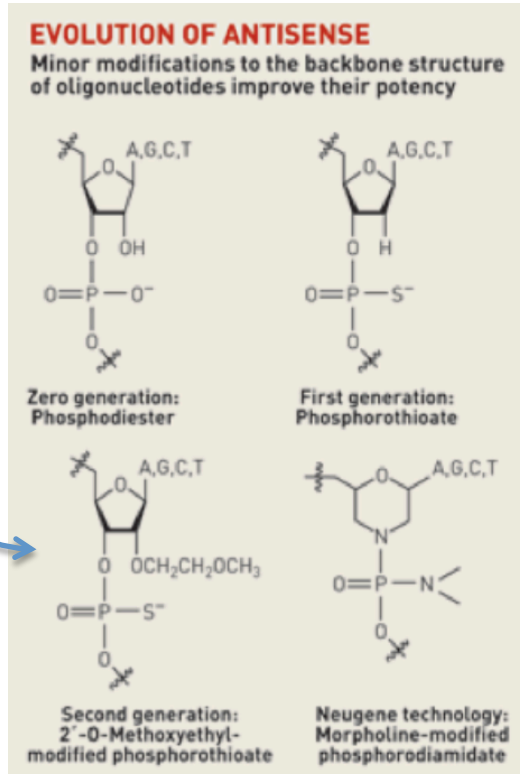


Fig. 1. Chemical structures of the oligonucleotides.

MOE is the favored form



Chemical & Engineering News: Cover Story - Back In The Game
Long the target of skepticism, oligonucleotide-based antisense drugs are starting to regain favor with investors
http://pubs.acs.org/email/cen/html/041706154028.html

Nathalie Dias and C. A. Stein. Antisense Oligonucleotides: Basic Concepts and Mechanisms. Molecular Cancer Therapeutics. Vol. 1, 347–355, March 2002

"MOE gapper"

ASO Screening at ISIS

ASO screens not only are designed to identify sequence but also most effective combination of base chemistry.

The most basic screen ISIS conducts is one plate which holds 79 ASOs and evaluate multiple target sites only.

Complex screens can be up to 1640 ASOs (21 plates) to evaluate multiple target sites and chemistries.

Lead compounds (66 for HTT) are selected and evauated for target knockdown in mice.

ASO lead follow-up

The most promising lead is selected for further evaluation

Test in transgenics

Screen liver transaminase as a biomarker for safety

Test the safest ones in transgenics with a dose response

Test tolerability in rats

Test in monkeys

Our Phase I ASO screen

ISIS will conduct a screen of 79 ASOs blocking human ATXN2 gene

We should expect 12 ASOs for evauation in cells

We want to reduce to 1-3 oligos for further testing

Our Phase II ASO screen

ISIS will conduct 3 screens of larger numbers of the following:

- ASOs against human ATXN2
- ASOs against mouse ATXN2
- ASOs against human and mouse ATXN2

ASO function and ASOs for CAG repeat proteins

- The published so-called "allele specific" ASOs targeting expanded CAG repeat genes also reduce wildtype some (Gagnon et al (David Corey) Biochem 49:2010).

- Such ASOs are expected to also target other CAG repeat genes so must be thoroughly evaluated for specificity.

- ASO function:

  ASOs bind the target RNA and the presence of the DNA-RNA heteroduplex stimulates RNase H to degrade the RNA strand

  For this reason ASOs must have high in vivo stringency because off-target binding can lead to degradation of the wrong RNAs. ISIS claims to have solved this problem primarly through informatics. Testing for off-target effects is difficult.

- ASOs targeting CAG repeats do not elicit RNase H activity. So its thought that these function by blocking translation. Such ASOs work best when the CAG repeat is close to the start codon. (might be ASOs targeting CAGs downstream work differently).

- ASOs targeting RNA made from cDNA do not seem to work. ASOs targeting preRNA work best for eliciting RNase H activity. Thus we can not expect to test ASOs in our regular transgenic animals. We have to use the BAC mice.

Gagnon et al

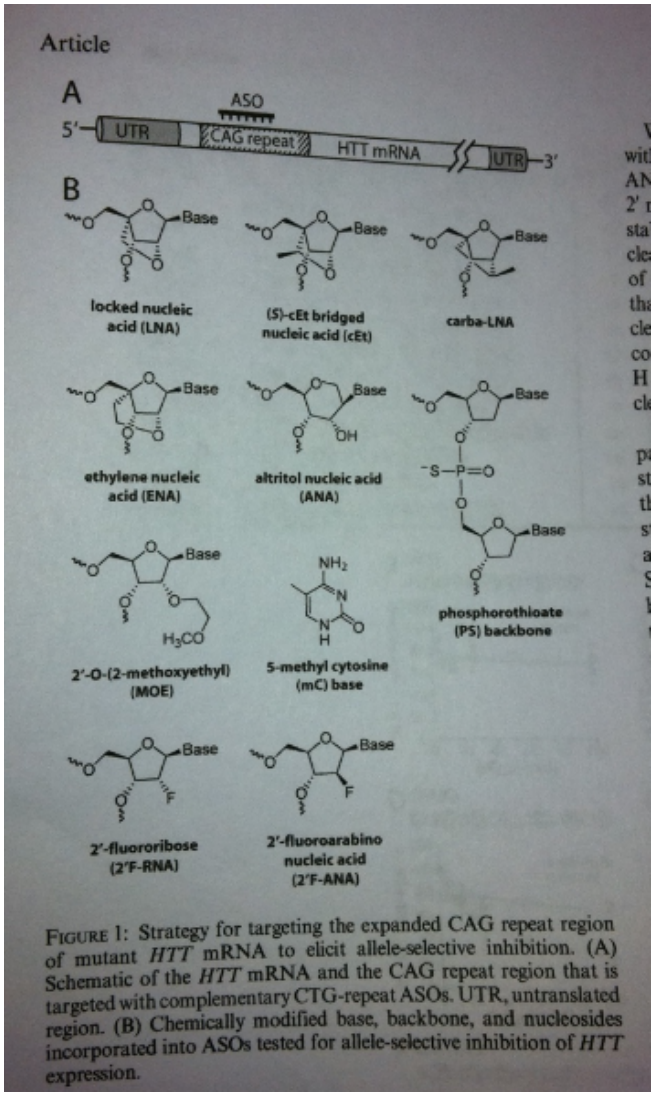
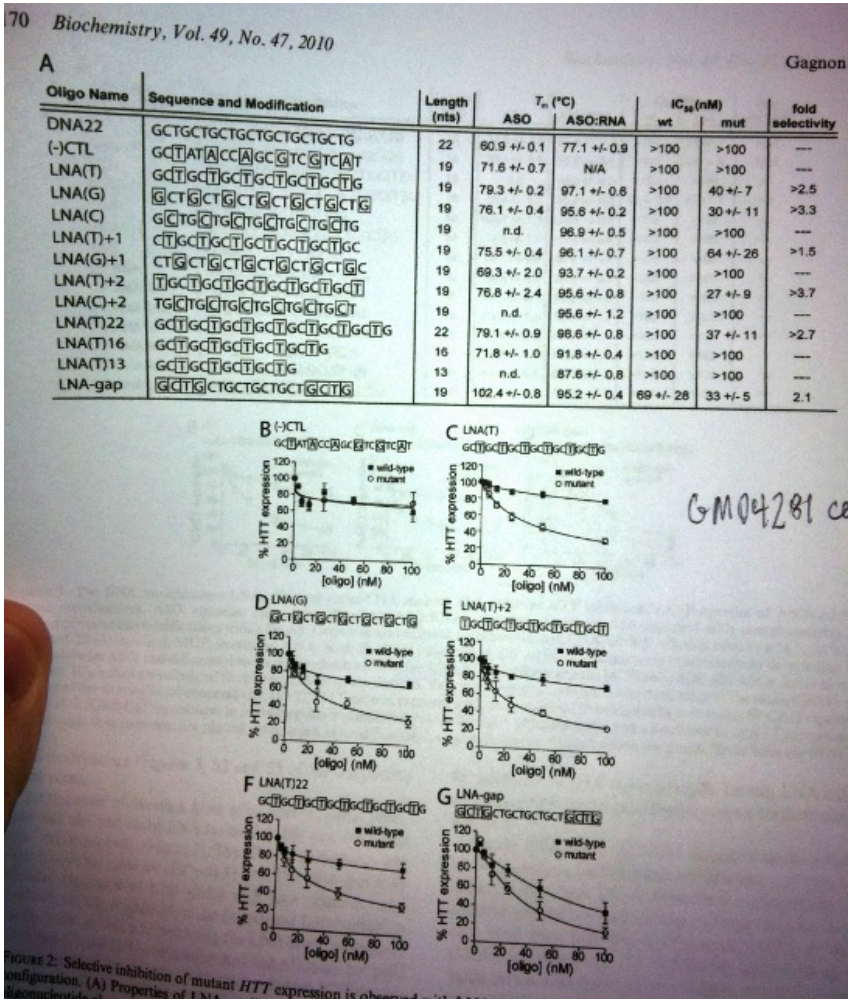


FIGURE 1: Strategy for targeting the expanded CAG repeat region of mutant *HTT* mRNA to elicit allele-selective inhibition. (A) Schematic of the *HTT* mRNA and the CAG repeat region that is targeted with complementary CTG-repeat ASOs. UTR, untranslated region. (B) Chemically modified base, backbone, and nucleosides incorporated into ASOs tested for allele-selective inhibition of *HTT* expression.



70 *Biochemistry, Vol. 49, No. 47, 2010*

A

Gagnon

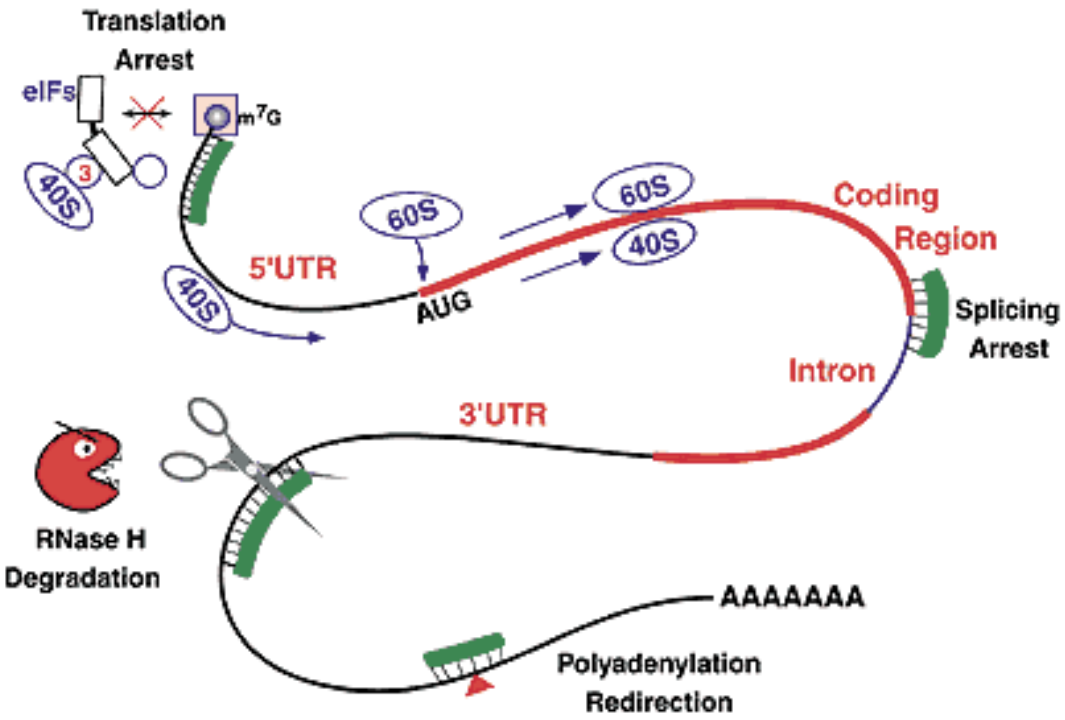
Oligo Name	Sequence and Modification	Length (nts)	T_m (°C) ASO	T_m (°C) ASO:RNA	IC_{50} (nM) wt	IC_{50} (nM) mut	fold selectivity
DNA22	GCTGCTGCTGCTGCTGCTG	22	60.9 +/- 0.1	77.1 +/- 0.9	>100	>100	---
(-)CTL	GCTATACCAGCGTCGTCAT	19	71.6 +/- 0.7	N/A	>100	>100	---
LNA(T)	GCTGCTGCTGCTGCTGCTG	19	79.3 +/- 0.2	97.1 +/- 0.6	>100	40 +/- 7	>2.5
LNA(G)	GCTGCTGCTGCTGCTGCTG	19	76.1 +/- 0.4	95.6 +/- 0.2	>100	30 +/- 11	>3.3
LNA(C)	GCTGCTGCTGCTGCTGCTG	19	n.d.	96.9 +/- 0.5	>100	>100	---
LNA(T)+1	CTGCTGCTGCTGCTGCTGC	19	75.5 +/- 0.4	96.1 +/- 0.7	>100	64 +/- 26	>1.5
LNA(G)+1	CTGCTGCTGCTGCTGCTGC	19	69.3 +/- 2.0	93.7 +/- 0.2	>100	>100	---
LNA(T)+2	TGCTGCTGCTGCTGCTGCT	19	76.8 +/- 2.4	95.6 +/- 0.8	>100	27 +/- 9	>3.7
LNA(C)+2	TGCTGCTGCTGCTGCTGCT	19	n.d.	95.6 +/- 1.2	>100	>100	---
LNA(T)22	GCTGCTGCTGCTGCTGCTGCTG	22	79.1 +/- 0.9	96.6 +/- 0.8	>100	37 +/- 11	>2.7
LNA(T)16	GCTGCTGCTGCTGCTG	16	71.8 +/- 1.0	91.8 +/- 0.4	>100	>100	---
LNA(T)13	GCTGCTGCTGCTG	13	n.d.	87.6 +/- 0.8	>100	>100	---
LNA-gap	GCTGCTGCTGCTGCTGCTG	19	102.4 +/- 0.8	95.2 +/- 0.4	69 +/- 28	33 +/- 5	2.1

GM04281 ce

FIGURE 2: Selective inhibition of mutant *HTT* expression is observed [illegible] configuration. (A) Properties of LNA [illegible] oligonucleotide

Can we get SCA2 patient fibroblasts for ASO evaluation??

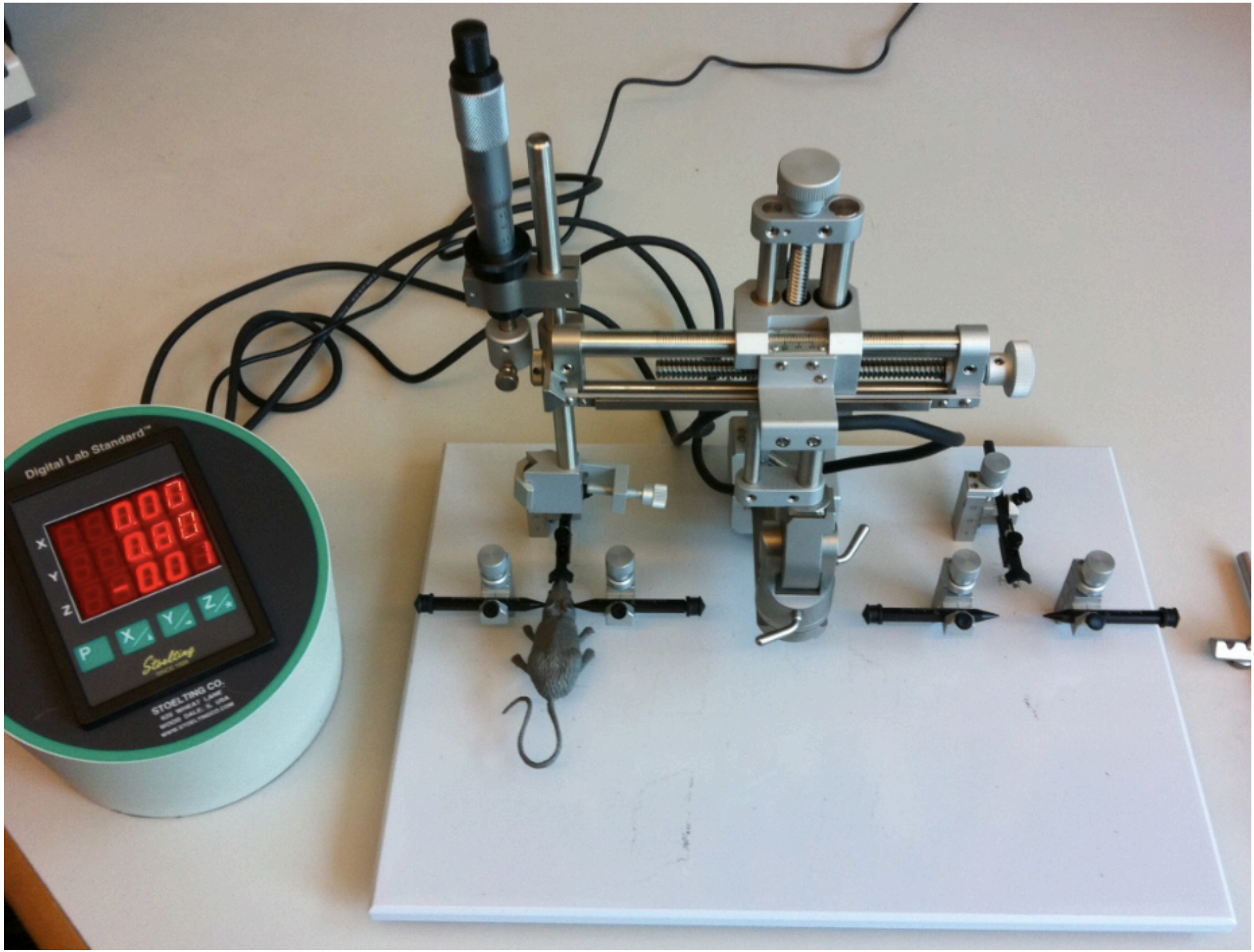
ASO actions on the pre-mRNA

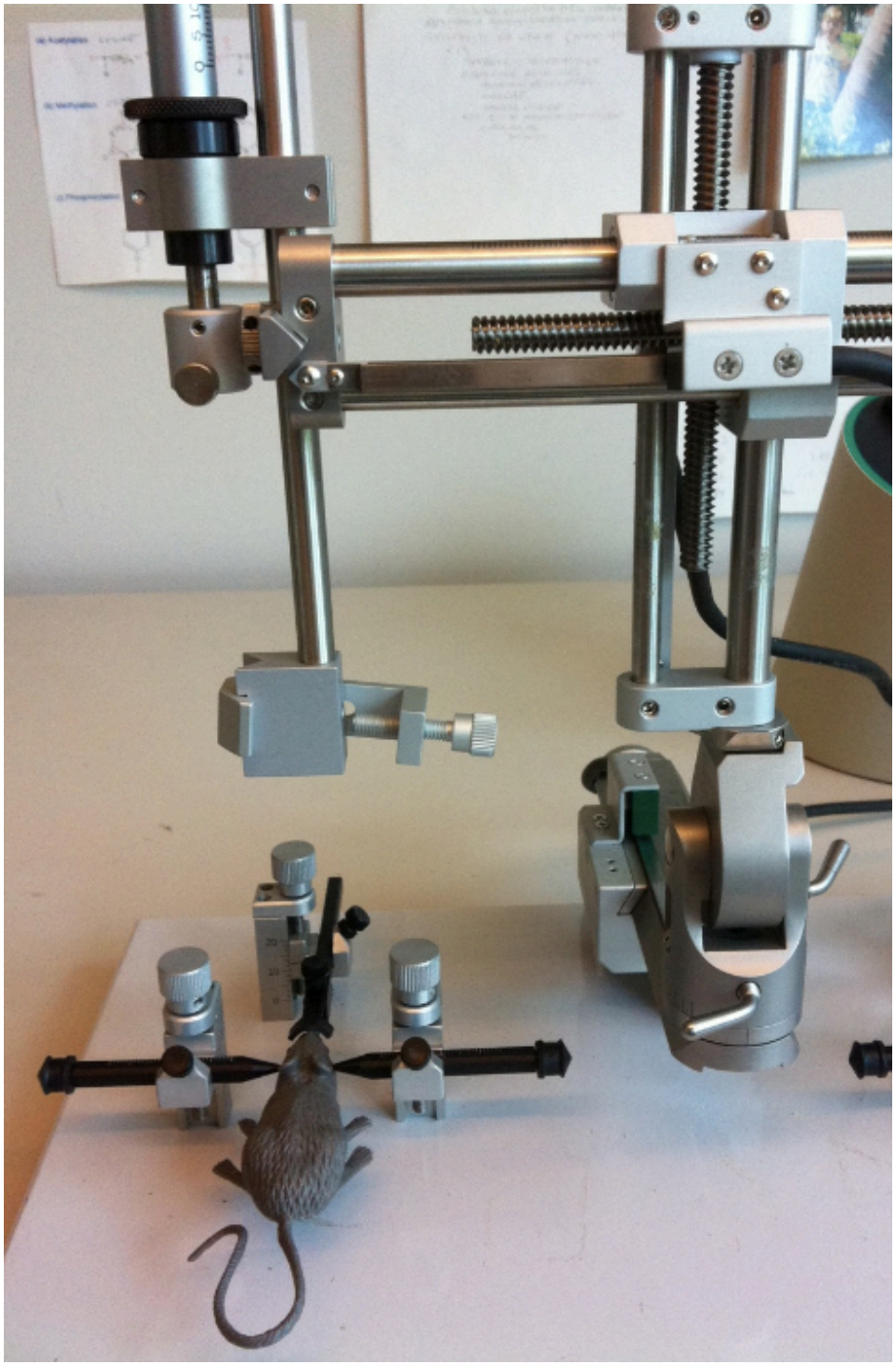


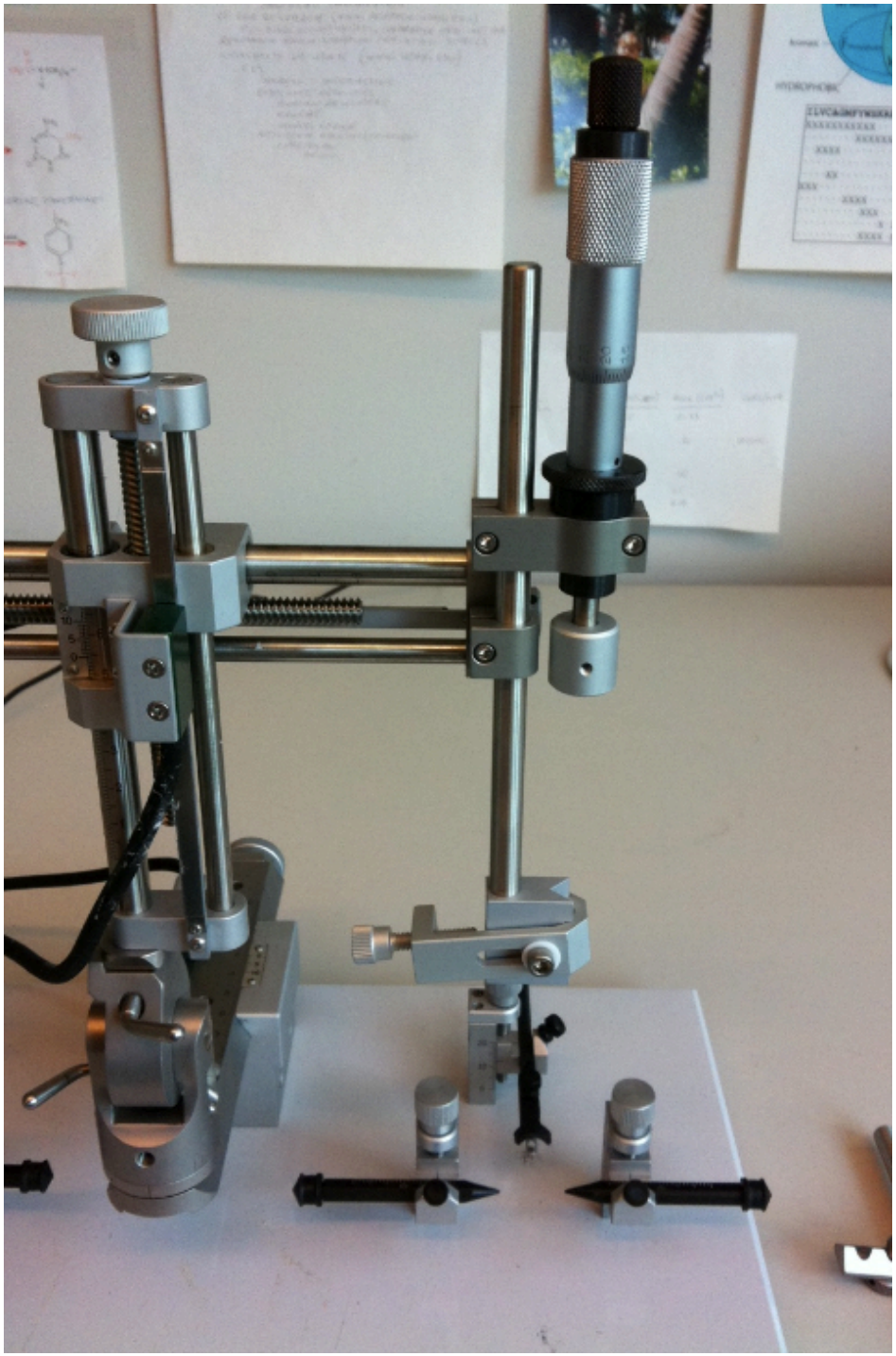
Drug News Perspect 2001, 14(8): 453

CAG repeat is involved in translation initiation ??

Duong’s Stereotaxic Instrument


Digital Lab Standard™
X
Y
Z
P
Stoelting
STOELTING CO.





ASI $3850

Home :: Stereotaxic :: SAS-4100 Small Animal Stereotaxic

Stereotaxic

Product 1 / 2

prev listing next

larger image

SAS-4100 SMALL ANIMAL STEREOTAXIC INSTRUMENT

$3,850.00

The SAS-4100 comes complete with a durable corian base, U-frame design, one MM-4100 three axis micromanipulator (choice of right or left handed), one standard electrode holder, choice of rat or mouse adapter, & ear bars.

- Model: SAS-4100

Add to Cart: 1

Product 5 / 8

prev listing next

larger image

RA-100G ANESTHESIA MASK ADAPTER

$475.00

Anesthesia Mask Adapter for Adult Mouse or Rat

- Model: RA-100G

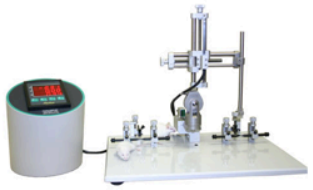
Add to Cart: 1

http://texasscientific.net/products/index.php?main_page=index&manufacturers_id=2

Stoelting: $5975

Digital Just for Mice™ Stereotaxic Instrument

Two Stereotaxic Instruments in One



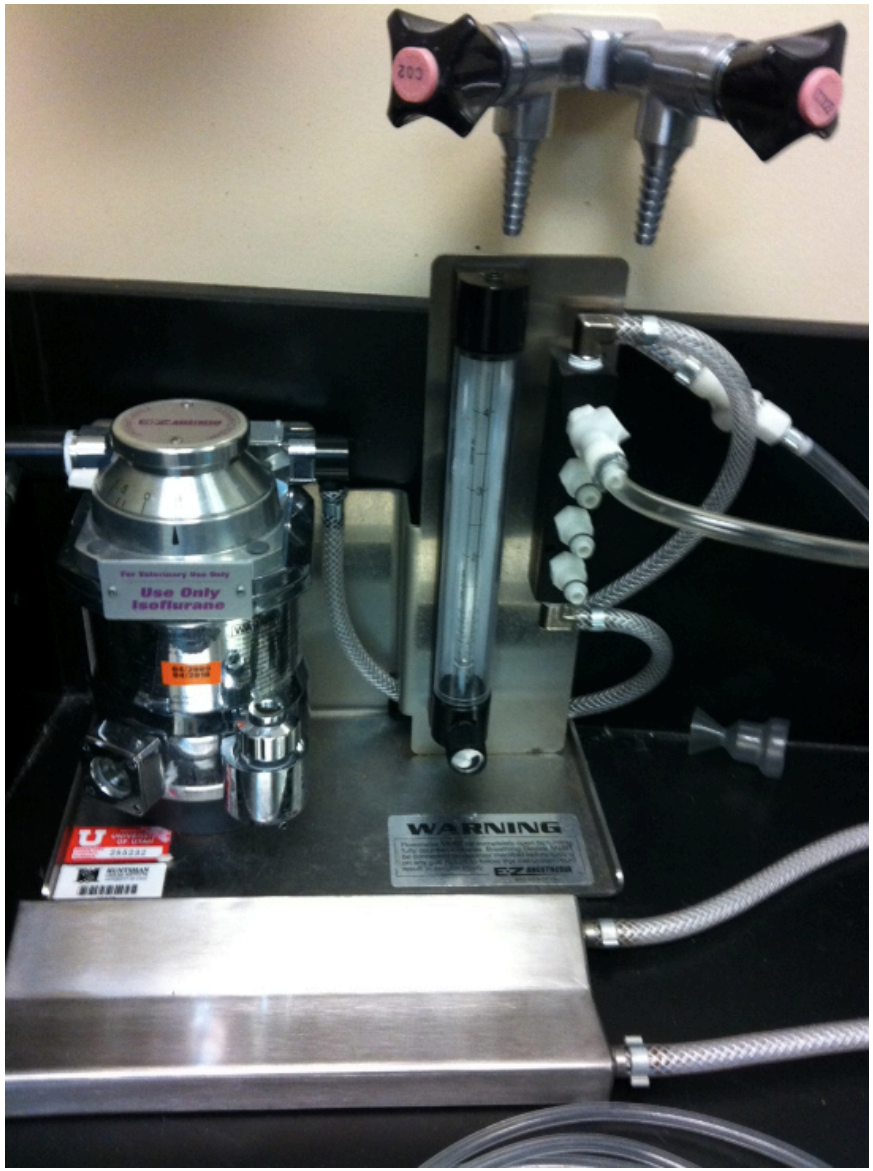
The Stoelting Just For Mice™ Stereotaxic Instrument was developed for the expanding growth of research being conducted with knock-out and transgenic mice. It allows the user to perform surgical procedures on two mice at one time. Precision alignment is accurate to 10 microns in all directions. Stoelting has created two stereotaxic instruments on a single base with ear bar slots on both sides. A manipulator arm controls medio-lateral and vertical positioning via lead screws, and antero-posterior movement via dovetail slide, with 80mm of travel possible in each direction, allowing access to both ear bar locations. Ear bars may be independently adjusted in height to level the skull. Delrin ear bars with tapered points, specialized jaw holder cuffs, or rubber pads are included.

Model	Description	Price
51725-D	Digital Just for Mice™	$5,975.00/Ea Add to Cart
Go to Cart		

Anesthesia Platform/Mouse Mask
$345

Stoelting Co
Customer Service: 800-860-9775
Tech support: 630-866-9700
loren@stoeltingco.com

Isoflurane Vaporizer



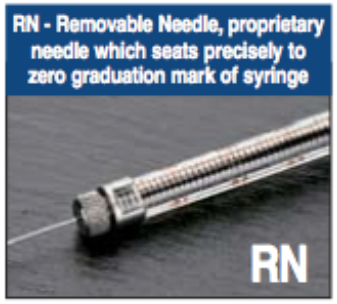
This is on the IVIS. Stephen Lessnick said we can use it on the stereotax

Hamilton Syringe

10 ul Hamilton Syringe
Part number 1701 with
detachable needle
26 gauge

Huber Point
(non-coring)

Point Style 2



1700 & 1800 Series Gastight Syringes	10 µl
	1701, 1801
Scale Length, cm	6
Total Scale Divisions	100
Major Graduations, µl	1
Minor Graduations, µl	0.2
Sub-Minor Graduations, µl	0.1

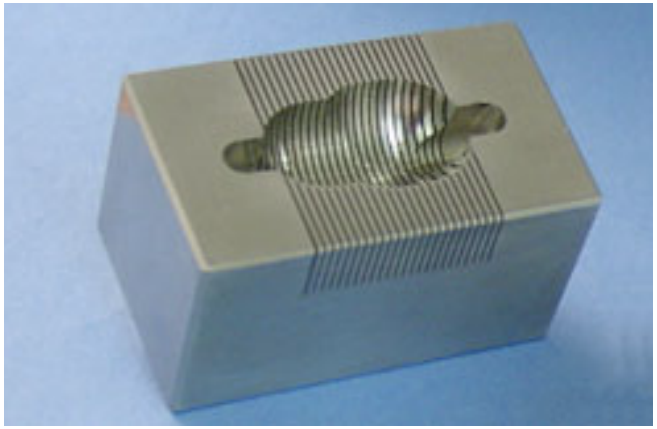
Reset

Category: 1700 Series Volume: 10 µL Termination: RN Point Style: 2

Found: 1 Products

Part Number	Description	Unit Price	Quantity	
80030	1701RN 10ul SYR (26s/2"/2)	48.00		Buy Now!

Dissection



From Plastics One

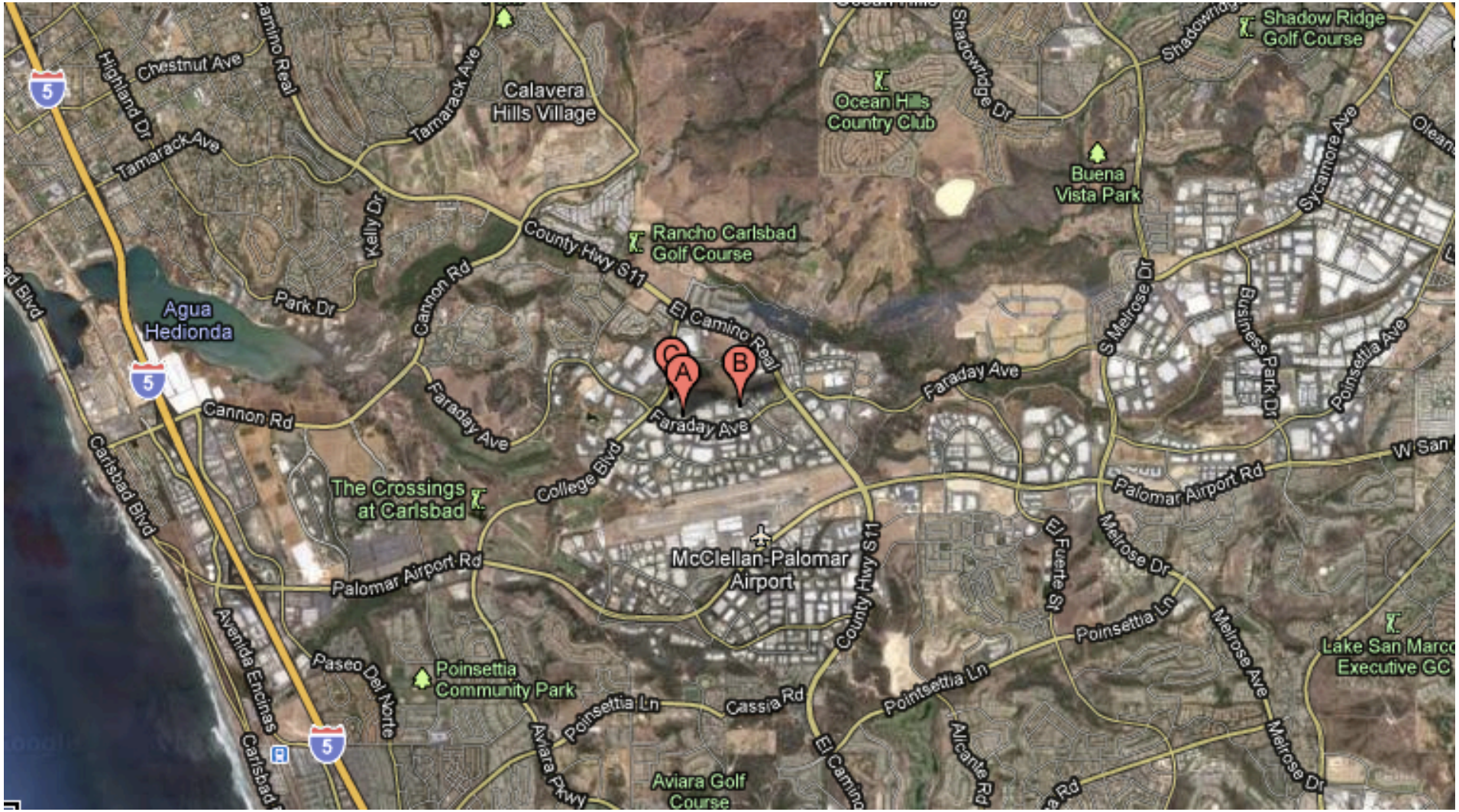
SBIR	NINDS SBIR	STTR
Phase I: $150,000 6 months	Phase I: $350,000/yr 2 years	Phase I: $100,000 1 year
Phase II: $1,000,000 2 years	Phase II: $1,000,000/yr 3 years (competing renewal allowed)	Phase II: $750,000 2 years
= $1.15 mil	= $3.35 mil +	= $0.85 mil

SBIR & STTR: Some of the NIH ICs offer the opportunity to submit Phase II Competing Renewal applications that will provide additional funding for Phase II SBIR projects. These renewals are often offered for those projects that require regulatory approval for the product or service being developed or to support complex instrumentation, clinical research tools, and behavioral interventions/treatments.

LIMITED AMOUNT OF AWARD

For budgetary, administrative, or programmatic reasons, NINDS may decrease the length of an award and/or the budget recommended by a review committee, or not fund an application. Generally, NINDS does not fund Phase I applications greater than $350,000 total cost per year for up to 2 years or Phase II applications greater than $1,000,000 total cost per year for up to 3 years. Applicants considering a requested budget greater than these limits are strongly encouraged to contact program staff before submitting an application.

ISIS PHARMACEUTICALS



Carlsbad Flower Fields













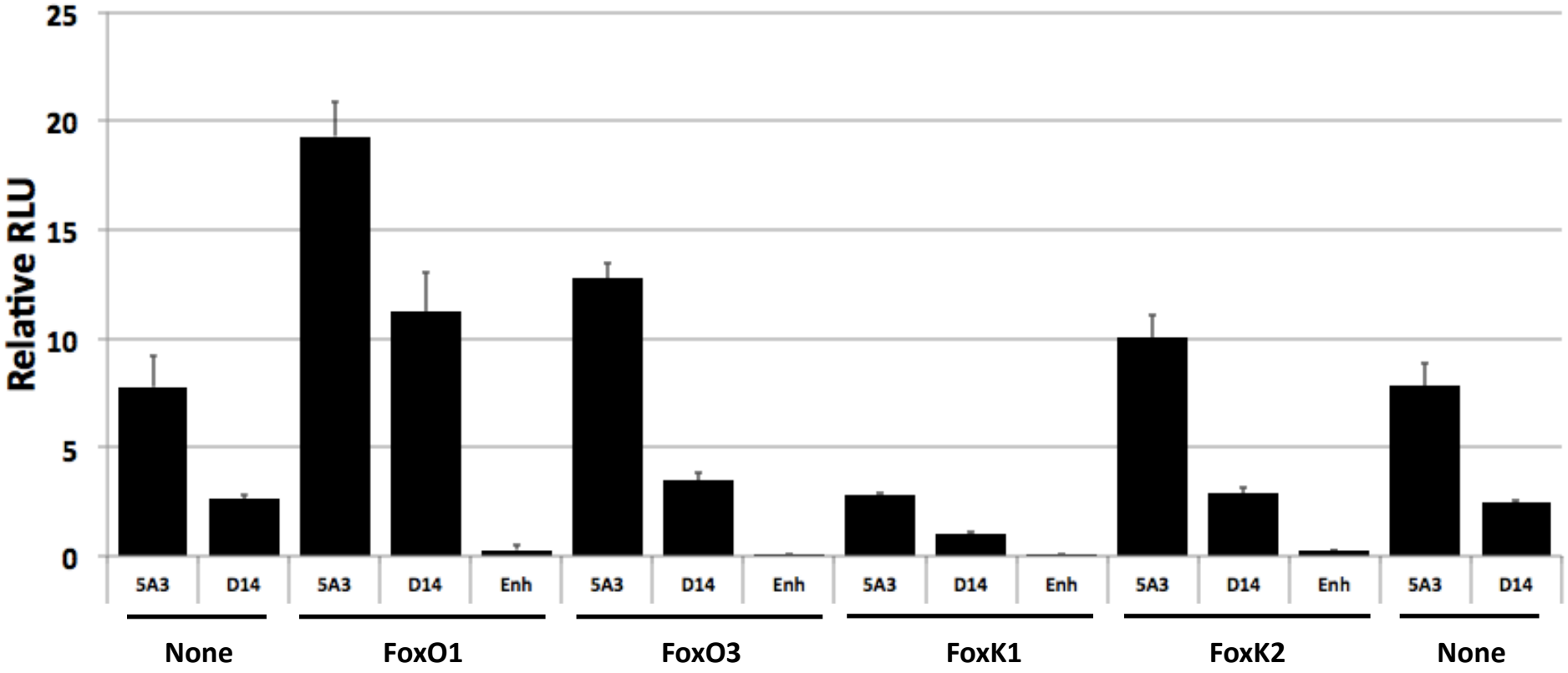


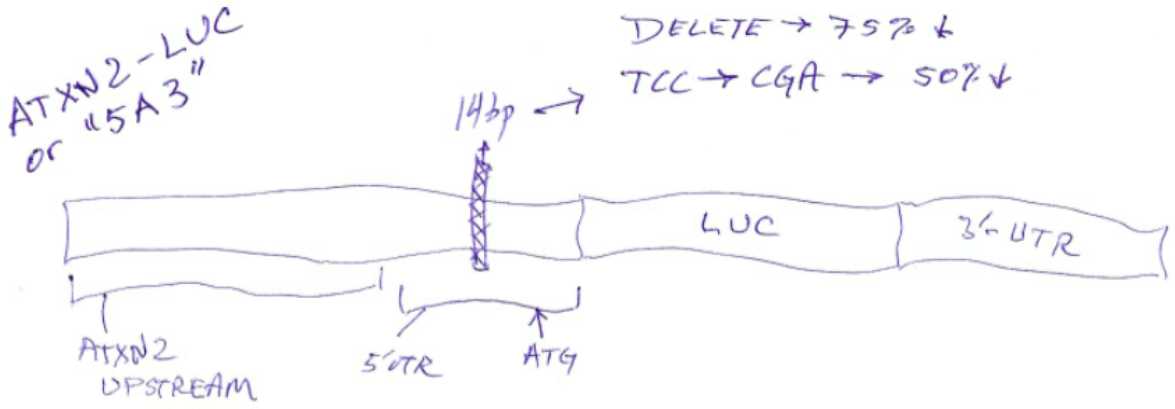

THE FLOWER FIELDS




Transcription factor paper

Retrial


Relative RLU
25
20
15
10
5
0
5A3
D14
5A3
D14
Enh
5A3
D14
Enh
5A3
D14
Enh
5A3
D14
Enh
5A3
D14
None
FoxO1
FoxO3
FoxK1
FoxK2
None


ATXN2-LUC
or "5A3"
DELETE → 75% ↓
TCC → CGA → 50% ↓
14bp
LUC
3'-UTR
ATXN2
UPSTREAM
5'UTR
ATG

Forkheads are characterized by GTAAACA

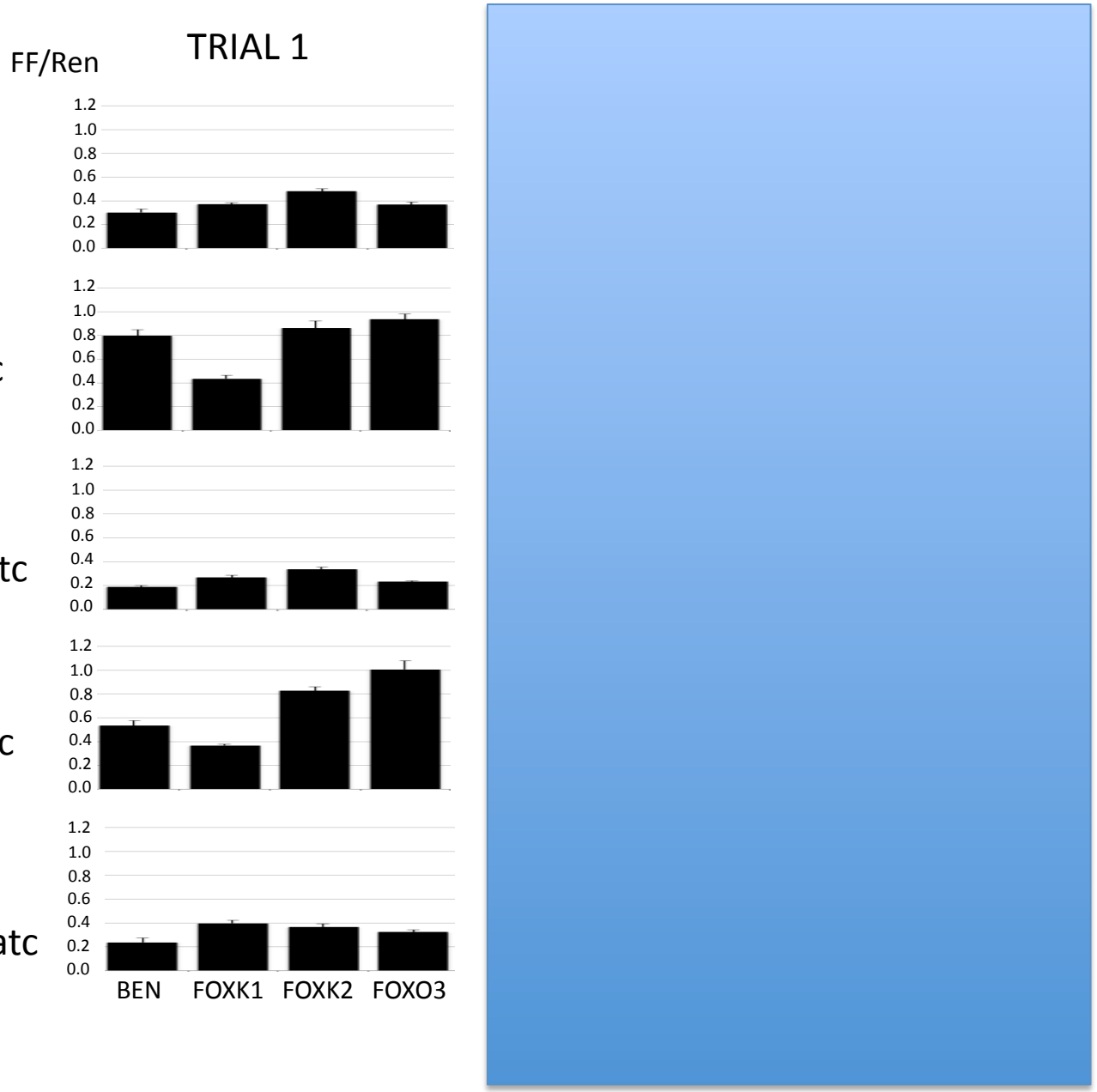
TABLE 1. *Described high-affinity binding sites of various Forkhead transcription factors*

FOX factor name	Name in original description	High-affinity binding site described								Original description (ref.)
FOXF2	FREAC-2	G/A	T	A	A	A	C/T	A	A	54
FOXC1	FREAC-3	G	T	A	A	A	C/T	A	A	54
FOXD1	FREAC-4	G/A	T	A/C	A	A	C	A	N	54
FOXL1	FREAC-7	G/A	T/C	A/C	A	A	C/T	A	N	54
FOXQ1	HFH-1	A/C	T	A	A	A	C	A	A/T	55
FOXD3	HFH-2	A/T	T	A	A	A	C	A	A/T	55
FOXA3	HNF-3	G/A	T/C	A/C	A	A	C/T	A	A/T	55
FOXI1	N/A	G/A	C	C	A	A	T	C/G	A	56
FOXK2	ILF-1	G	T	A	A	A	C	A	A	57
FOXO1	FKHR	G	T	A	A	A	C	A	A	58
FOXO3A	FKHRL1	G	T	A	A	A	C	A	A	58
FOXO4	AFX	G	T	A	A	A	C	A	A	58
FOXP1	N/A	A	T/C	A	A	A	C	A	A	35
FOXL2	N/A	G	T	C/G	A	A	G	G	T	5
Forkhead	General consensus	G/A	T/C	A/C	A	A	C/T	A	N	

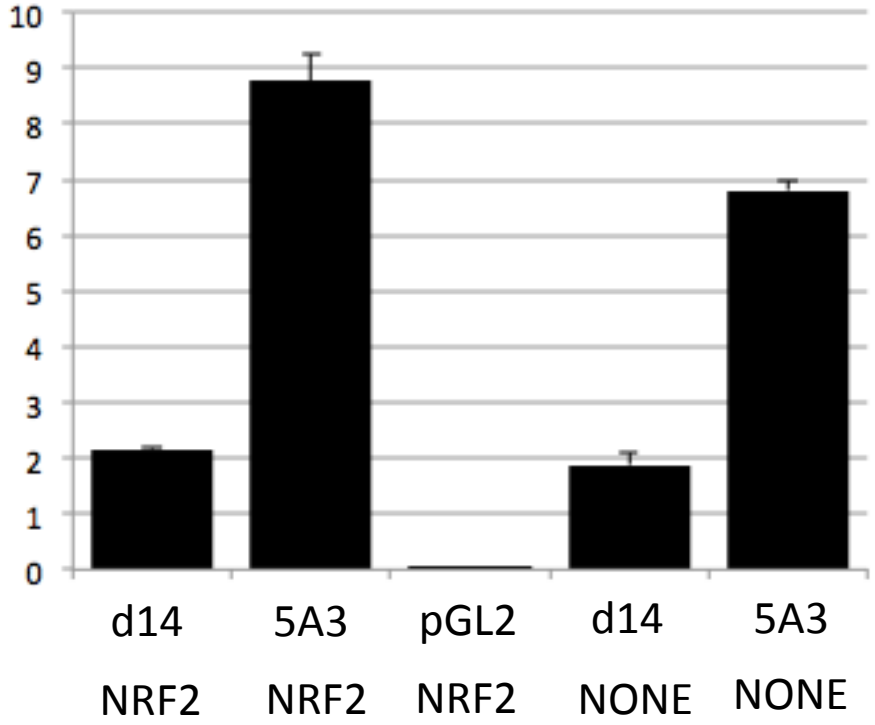
N/A, not available.

Georges et al., Generic binding sites, generic DNA-binding domains: where does specific promoter recognition come from? FASEB Journal: 24,246-56; 2010

TF action on Prolactin minimal promoter with *ATXN2* 27mer promoter frag



NRF2 action on ATXN2-luc



22% increase by NERF2 that is eliminated by d14

Transcription Element Search System

http://www.cbil.upenn.edu/tess/

[6 .. 17] of 17 3 | 5 | 7 | 9 | 11 | 13 | 15 | 17 | 19 | 21 | 23 | 25 | 27 | 29

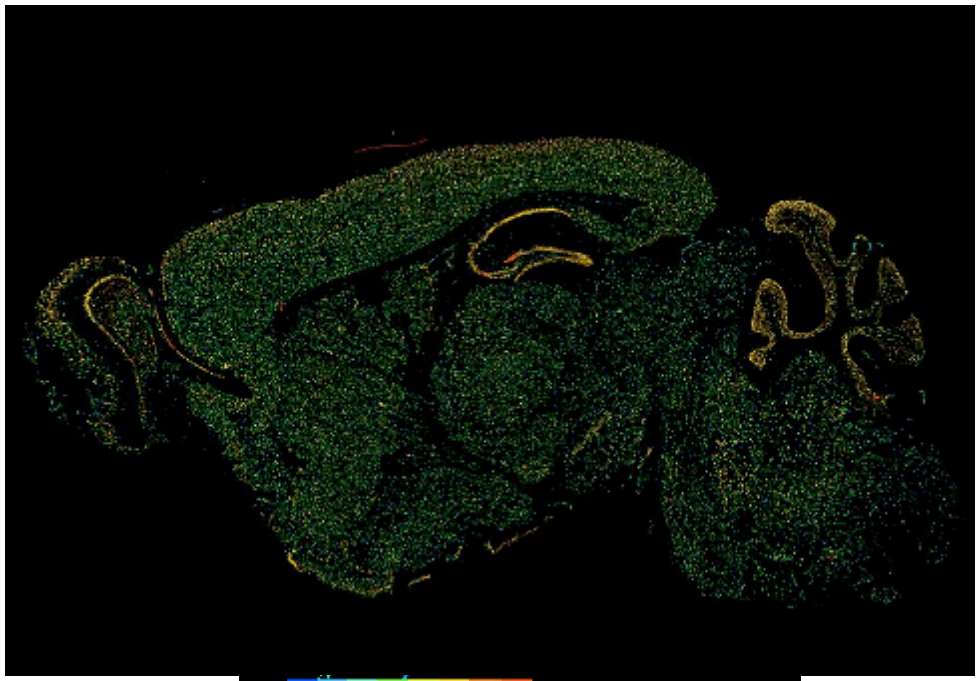
#	Factor	Model	Beg	Sns	Len	Sequence	L_a	$L_{a/}$	L_q	L_d	L_{pv}	S_c	S_m	S_{pv}	P_{pv}
6	T00714 RAF	R00256 ()	8	N	4	CCGA	8.00	2.00	1.000	0.00	nc	?	?	nc	nc
7	_00000 MAF	I00394 (MAF)	11	R	6	acttcc	10.23	1.71	1.000	0.00	1.6e-02	1.00	1.00	0.0e+00	nc
8	_00000 E1A-F	I00128 (E1A-F)	11	R	7	acttccg	12.07	1.72	1.000	0.00	3.8e-03	1.00	1.00	0.0e+00	nc
9	T00111 c-Ets-1 T00112 c-Ets-1 T00114 c-Ets-1 54 T00115 c-Ets-1 68 T00684 PEA3 T00685 PEA3 T00686 PEA3	R02153 ()	11	R	8	RCWTCCKS	12.00	1.50	1.000	0.00	nc	?	?	nc	nc
10	T01408 Fli-1	R04070 ()	11	R	8	ACTTCCKG	15.00	1.88	1.000	0.00	nc	?	?	nc	nc
11	T00111 c-Ets-1	Q00025 (-)	11	R	9	acttccggt	14.54	1.62	1.000	0.00	2.2e-04	1.00	1.00	0.0e+00	nc
12	_00000 f_alp-f_eps	I00006 (f_alp-f_eps)	12	R	7	cttccgg	11.09	1.58	1.000	0.00	3.8e-03	1.00	1.00	0.0e+00	nc
13	_00000 NRF-2	I00059 (NRF-2)	12	N	8	cttccggt	13.88	1.73	1.000	0.00	9.2e-04	1.00	1.00	9.2e-04	nc
14	T00975 Ttk_88K	Q00200 (-)	12	N	8	cttccggt	13.88	1.73	1.000	0.00	9.2e-04	1.00	1.00	0.0e+00	nc
15	T01059 MNB1a T02690 Dof2 T02691 Dof3 T02692 PBF	R08440 () R08441 () R08442 () R08443 ()	20	N	4	AAAG	8.00	2.00	1.000	0.00	nc	?	?	nc	nc
#	Factor	Model	Beg	Sns	Len	Sequence	L_a	$L_{a/}$	L_q	L_d	L_{pv}	S_c	S_m	S_{pv}	P_{pv}
16	T01214 NF-E	R00554 ()	29	N	5	CTATC	10.00	2.00	1.000	0.00	nc	?	?	nc	nc
17	T00305 GATA-1	R08167 ()	29	R	6	CTATCC	12.00	2.00	1.000	0.00	nc	?	?	nc	nc

NRF2

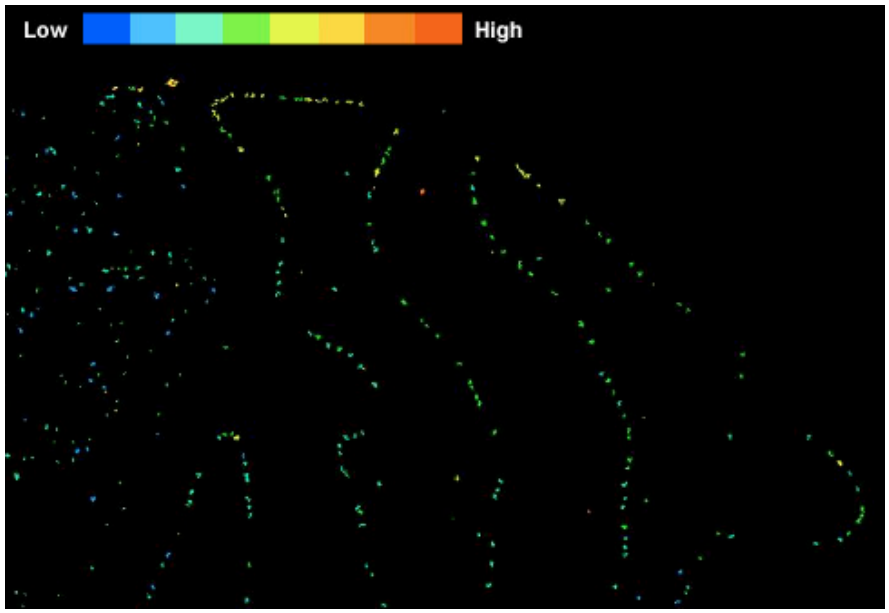
- NRF2 encoded by the *NRE2L2* gene (Nuclear factor erythroid-derived 2 like 2)
- "Master" regulator of the antioxident response important for ameliorating reactive oxidative stress
- bZLP family TF including NFE2, NFE2L1, NFE2L2 distinct from Jun and Fos
- When stress is absent NRF2 binds KEAP1 leading to ubiquitination and proteasomal degradation. NRF2 $t_{½}$ is 20 minutes when stress is absent. Unbound NRF2 translocates to the nucleus, binds MAF and the Antioxidant Response Element (ARE) activating antioxidative genes effective for reducing oxidative stress, inflammation and disease.
- Among NRF2 targets are the multidrug resistance-associated proteins (MRPS). NRF2 is therefore a target for cancer.

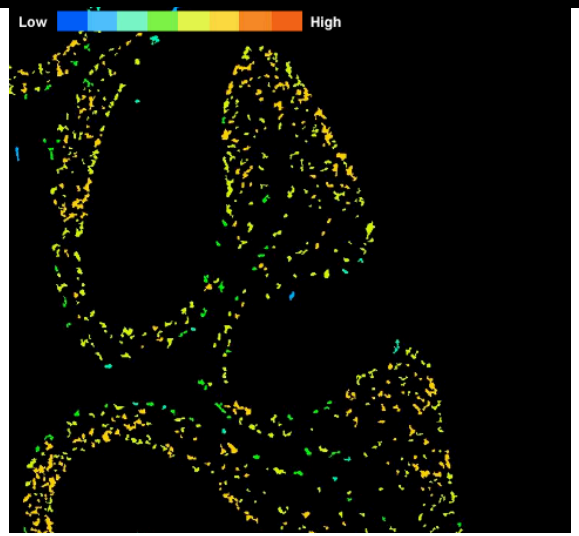
ATXN2 mutation→Stress→ NRF2 expression to combat stress→increased *ATXN2* expression

MAF1



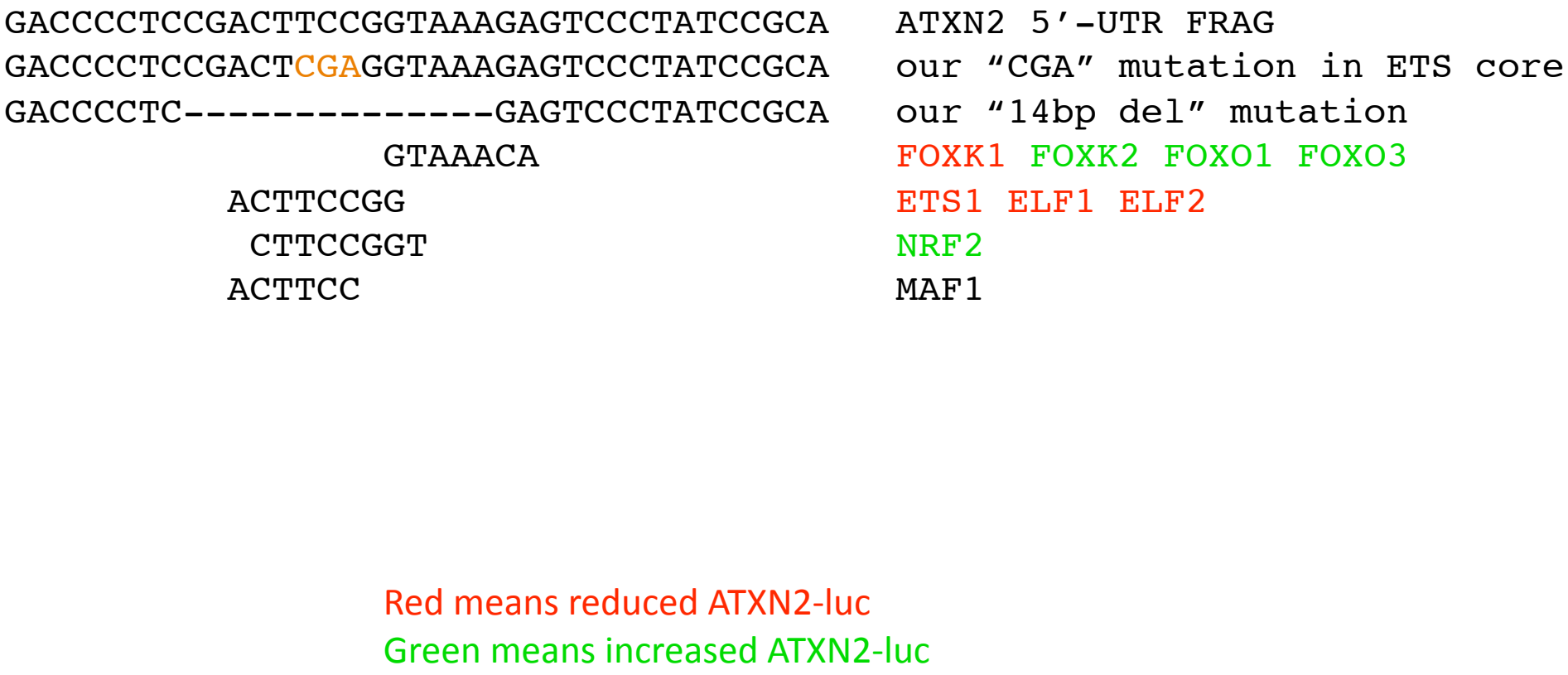
KEAP1





NRF2

- No great Allen Atlas pictures
- Shows present in cerebellum in Gene Cards
- Some literature shows NRF2 induction in cerebellum in response to stress.

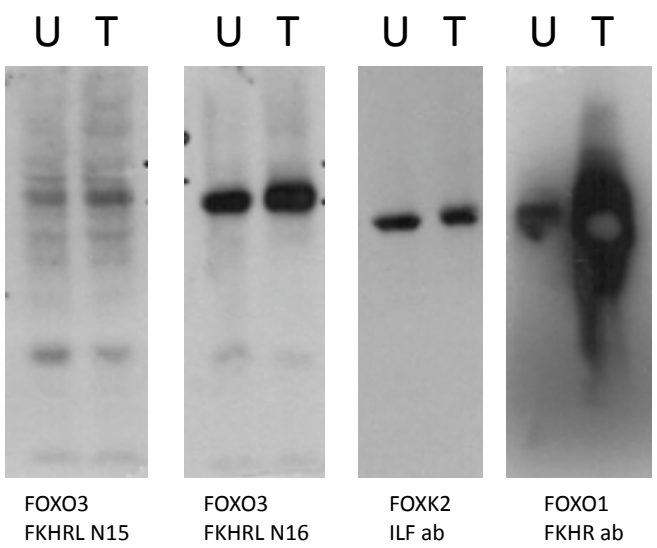


Red means reduced ATXN2-luc

Green means increased ATXN2-luc

Forkheads in HEK293 cells

Transfected (T)
Untransfected (U)



Tags: HA HA ? FLAG

Sharrocks didn't state what tag he gave me on FOXK2--his paper used 3xFLAG and GFP. I assume he gave me FLAG.

KK's 5/18/2011

How do we use this for therapy for SCA2?

Targeting individual TFs isn't feasible because we predict side-effects of some form of screwed-up-a-genesis

One way is to design a custom compound specifically blocking the TCC region.

> Pyrrolobenzodiazepine polyamide GWL-78 binds DNA preventing NF-Y binding: Kotecha et al., Mol Cancer Ther 7:1319;2008.
>
> Polyamides block DNA binding by HIF-1. Nickols et al., ACS Chem Biol. 2007 August 17; 2(8): 561–571.

Another is decoy hairpin ODNs

> Decoy ODNs trapping STAT3 or NFkB in cytoplasm: BMC Cell Biol. 2011 Apr 12;12:14.
>
> Others including one Methods Enz paper showing decoy ODN for sequestering CREB in suprachaismatic nucleus to alter circadian clock.

fin