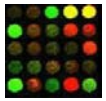
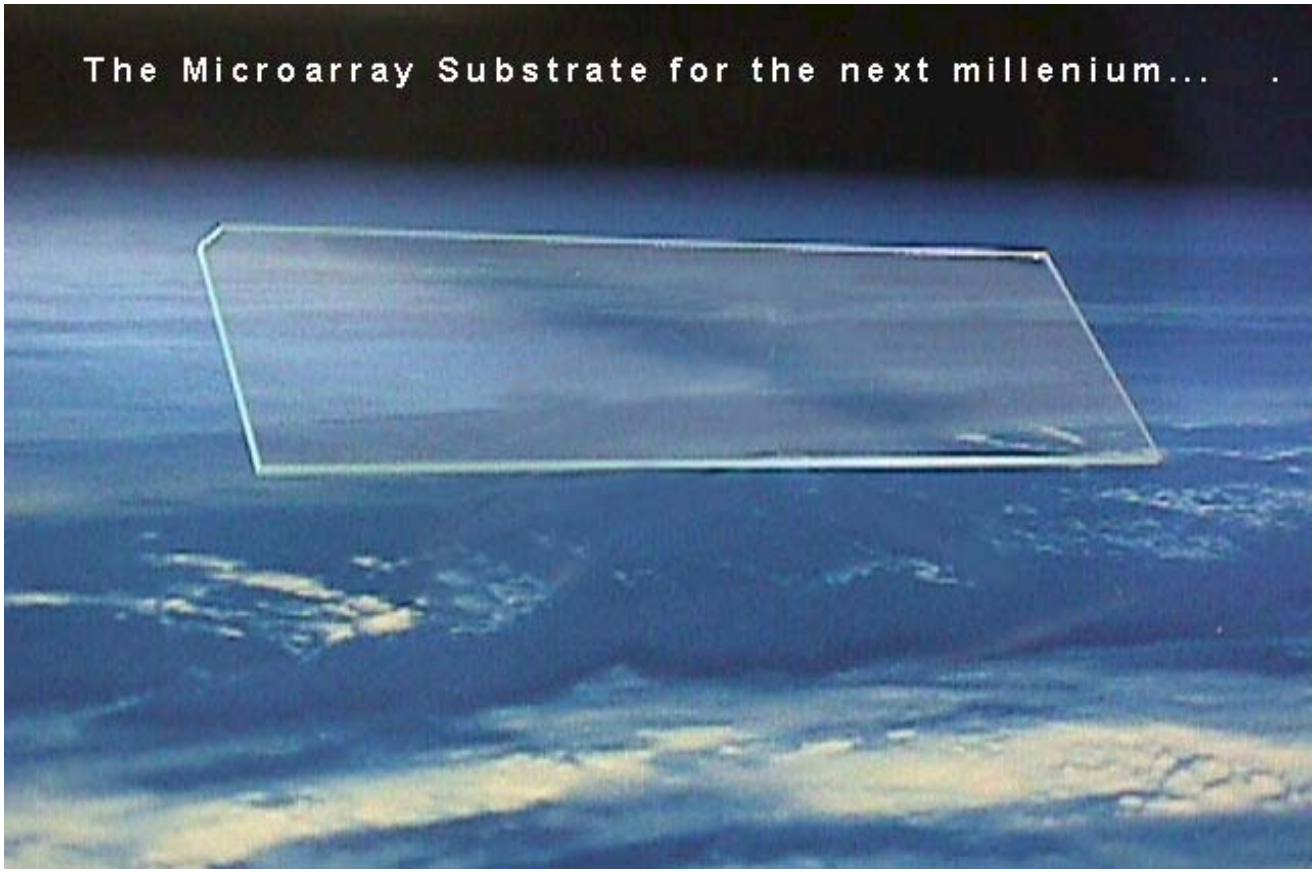
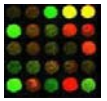


Identificazione di pathways molecolari associati alla resistenza all'imatinib nella leucemia mieloide cronica mediante la tecnologia del microarray. Dal design all'analisi dei dati

Rosanna Martinelli Ph.D.
Dipartimento di Biochimica e Biotecnologie Mediche
Università degli Studi di Napoli "Federico II"
CEINGE-Biotecnologie Avanzate

ICAR-CNR 18 dicembre 2007


The Microarray Substrate for the next millenium...



GENOME RESEARCH

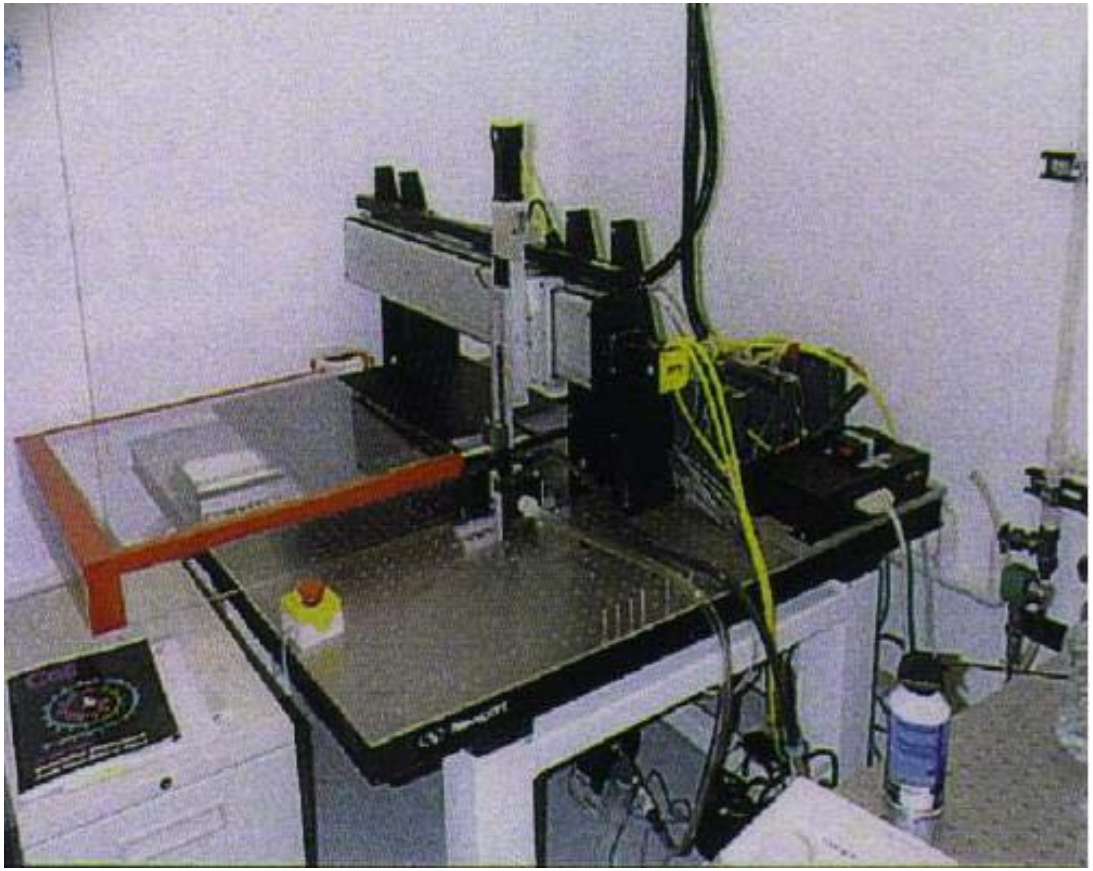
A DNA Microarray System for Analyzing Complex DNA Samples Using Two-color Fluorescent Probe Hybridization

Dari Shalon,[1,4] Stephen J. Smith,[3] and Patrick O. Brown[1,2,5]

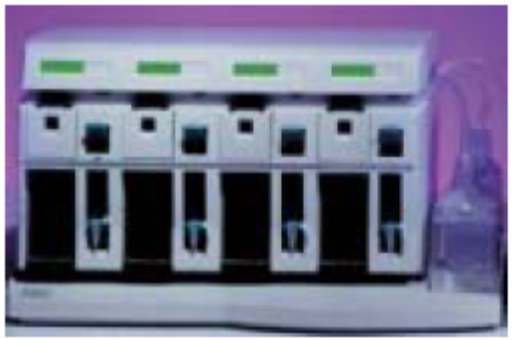
[1]Howard Hughes Medical Institute and Departments of [2]Biochemistry and [3]Molecular and Cellular Physiology, Stanford University, Stanford, California 94305

Genome Res. 1996 6: 639-645

THE FIRST ROBOT



Commercial MicroArrays



Fluidic station - Affymetrix

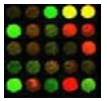
Affymetrix

Scanner

Hybridization ovens
– Agilent, Codelink

Agilent

MICROARRAY :NOT JUST FOR GENOMICS

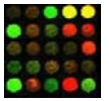


Although the sample types, detection methods and number of samples change, the biological assays themselves traditionally have remained

"GRIND AND BIND"

studying the binding events of various biomolecules to obtain informations about biology of a cell.

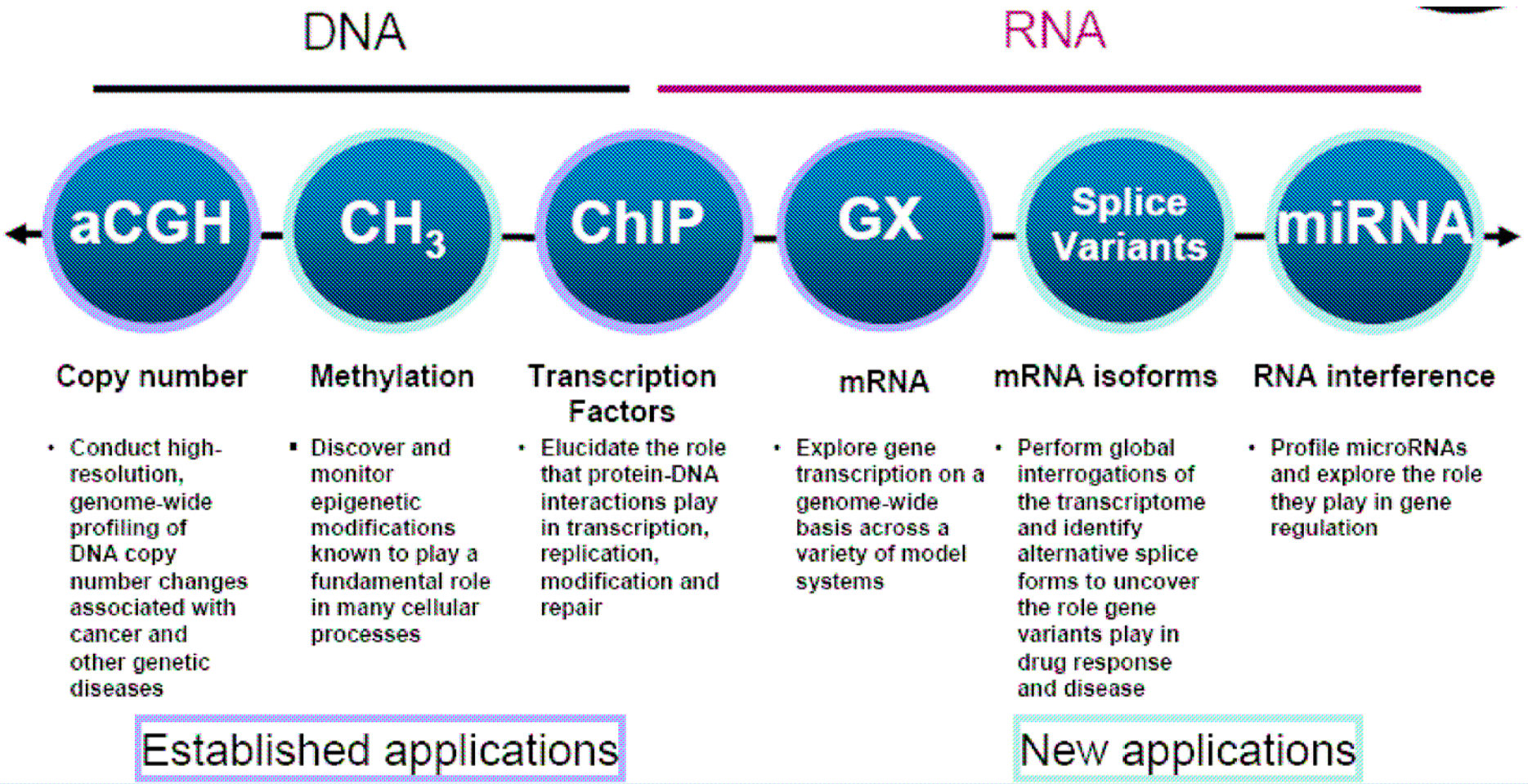
EX SITU MICROARRAY APPLICATIONS



SAMPLE TYPE	INTERACTION	APPLICATIONS
Oligonucleotide	DNA-DNA, DNA-RNA	Evolution, Gene Expression, Genotyping, Mapping, SNP Mapping,
(DNA, RNA)	RNA-RNA, DNA-Protein	SNP Detection, Structure-function, Two hybrid analysis
	RNA-Protein	
PCR Products	DNA-DNA, DNA-RNA	Evolution, Gene Expression, Genotyping, Mapping, SNP Detection,
	DNA-Protein	Structure-function,Two hybrid analysis
BACs	DNA-DNA, DNA-RNA	Contig assembly, Evolution, Genotyping, Mapping, Systemic Analysis,
	DNA-Protein	Comparative Genomic Hybridization
Protein	Protein-DNA, Protein-RNA	Drug Discovery, Epitope Mapping, Evolution, Gene Expression, Genoty
	Protein-Protein, Protein-Receptor	Proteomics, Post-Translational Analysis, Structure-Function,
	Protein-Small Molecule	Two Hybrid Analysis
Antibodies	Antibody-Antigen,	Epitope Mapping, Evolution, Gene Expression, Genotyping,
	Antibody-RNA	Proteomics, Post-Translational Analysis, Structure-Function,
	Antibody-DNA,	
	Antibody-Cell Surface	
	Antibody-Organelle	
Enzymes	Enzymes-Substrate	Kinetics, Substrate Specificity, Inhibitor Analysis
	Enzymes-Effector	
	Enzymes-Inhibitor	
Carbohydrates	Sugar-Protein, Sugar-Antibody	Docking, Signaling
	Sugar-Receptor	
Small Molecule	Small Molecule-Protein	Binding Kinetics, Drug Discovery

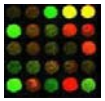
ceinge BIOTECNOLOGIE AVANZATE s.c.a.r.l.

Genomic Applications



From Agilent

Gene Expression:Each Platform has its own Advantages and Disadvantages,.



cDNA Microarrays

Advantages	Disadvantages
Increased detection sensitivity due to longer sequences	Cross-hybridisation can be a problem
Affordable	Maintaining clones and PCR amplification is labour-intensive

Oligonucleotide arrays (in general)

Advantages	Disadvantages
Higher specificity	Decreased sensitivity due to shorter sequences (lower sensitivity compensated by use of multiple probes)
More uniform melting temperatures	Expensive to design and manufacture
Detection of SNP and splice variants	
Higher uniformity of spots (controlled amount of material per spot)	

Affymetrix and Agilent Oligonucleotide arrays

Affymetrix

Only 1-colour is offered

Decreased sensitivity due to shorter sequences (25-mer); however, lower sensitivity compensated by the fact that multiple oligos represent each gene

Additional equipment (specialised fluidics station and Affymetrix-specific scanner) required

Agilent

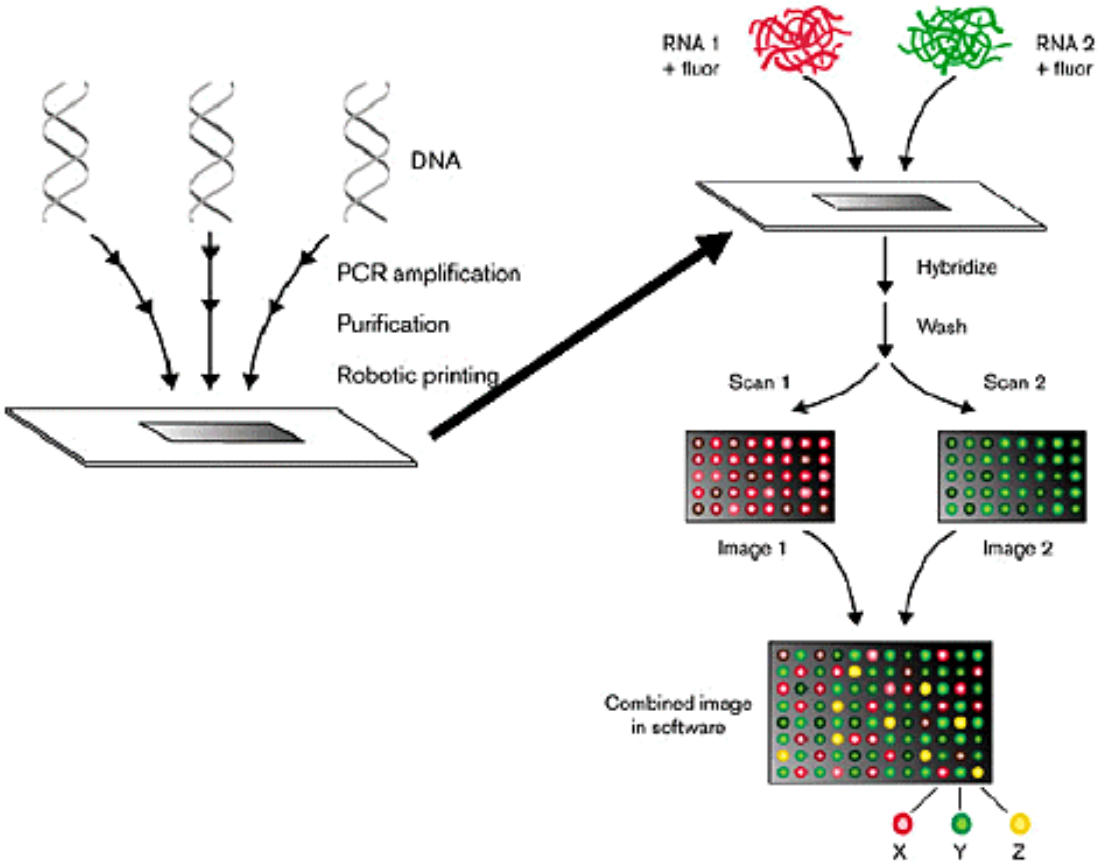
Offers both 1- and 2-colour systems

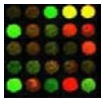
Longer 60-mers are more tolerant of sequence mismatches and thus are more suitable for analysis of highly polymorphic regions

Agilent 1-colour versus 2-colour

- 1-colour allows for experimental design to change throughout project – good for projects that will take place over a long period of time with an unknown number of samples
- 2-colour is a more direct comparison of two samples

ORIGINAL MICROARRAY FLOW-CHART

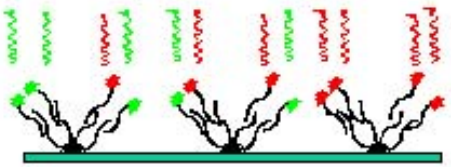




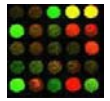
Two colour hybridisations:

A mixture of two DNA targets is hybridised to the chip

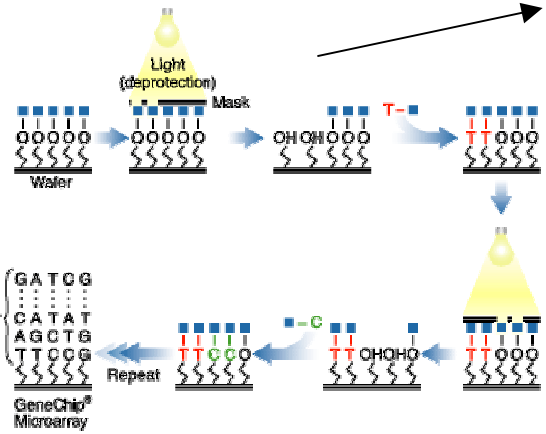
One is used as an internal control to enable more exact quantification



AFFYMETRIX



GeneChip



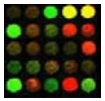
PHOTOLITOGRAPHY

HYBRIDIZATION OVEN

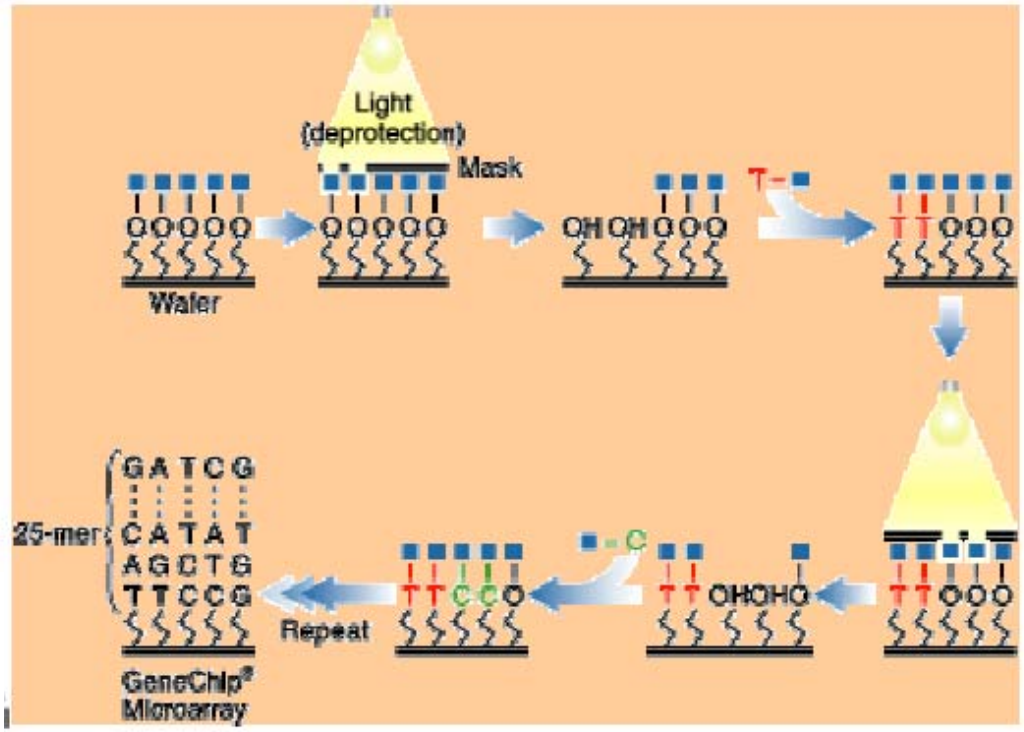
FLUIDICS STATION

SCANNER SYSTEM

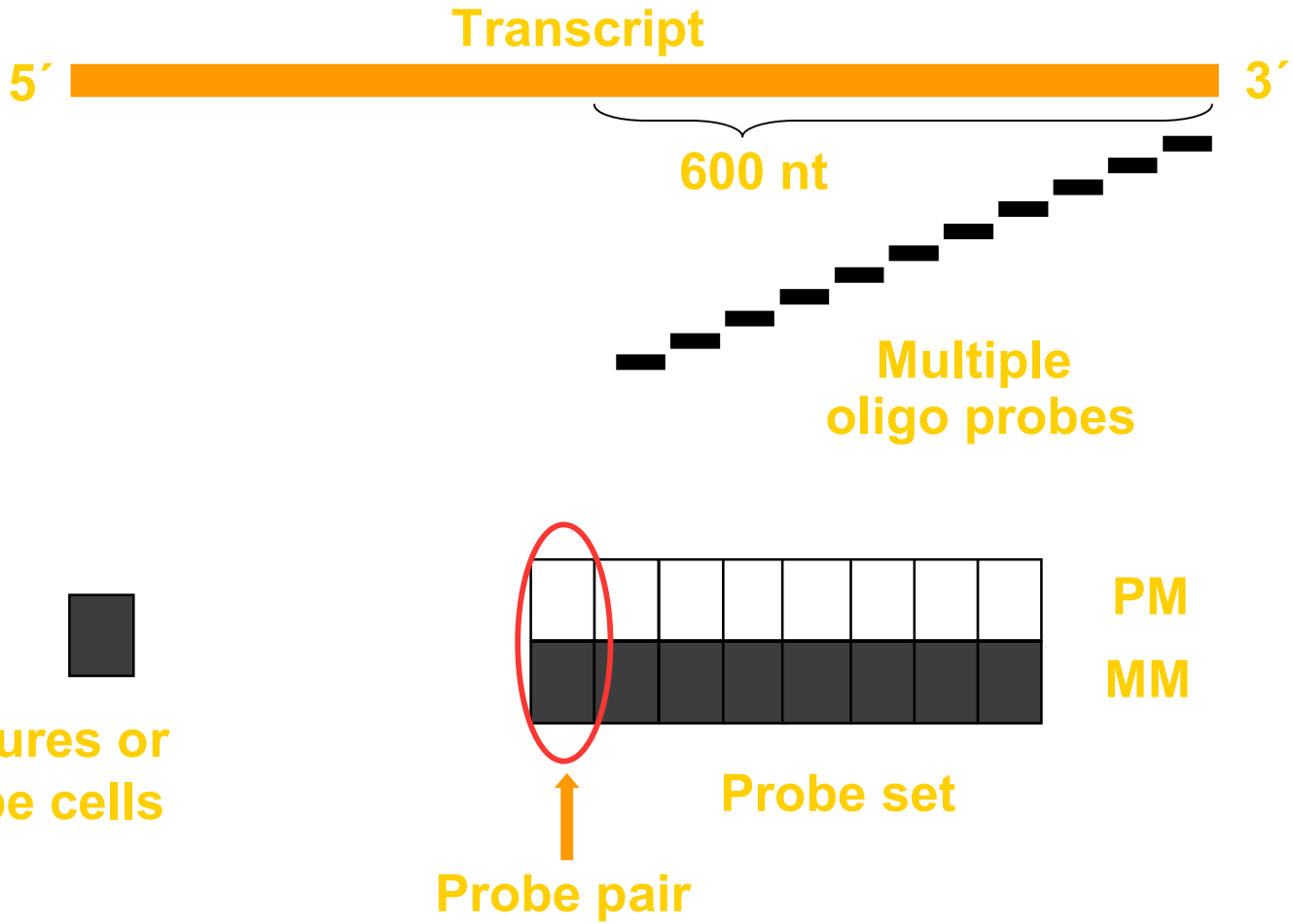
COMPUTER WORKSTATION



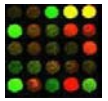
Oligonucleotide GeneChips using Photolitography and Combinatorial Chemistry



GeneChip® Expression Array Design



IMPORTANT STEPS IN MICROARRAY EXPERIMENTS



EXPERIMENTAL DESIGN

Goal(s) formulation
Resources allocation
Experimental unit definition
Statistical power and sampling
Statistical design validation

TARGETS COLLECTION

Selection
Characterization
Purification
QC

HYBRIDIZATION

Probes and slides treatment
Target labeling
QC
Hybridization
Washing
Drying

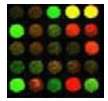
DATA TRANSFORMATION

Image acquisition
Signal quantification
Background subtraction
Flag
Replicates
Normalization
Statistical evaluation

KNOWLEDGE

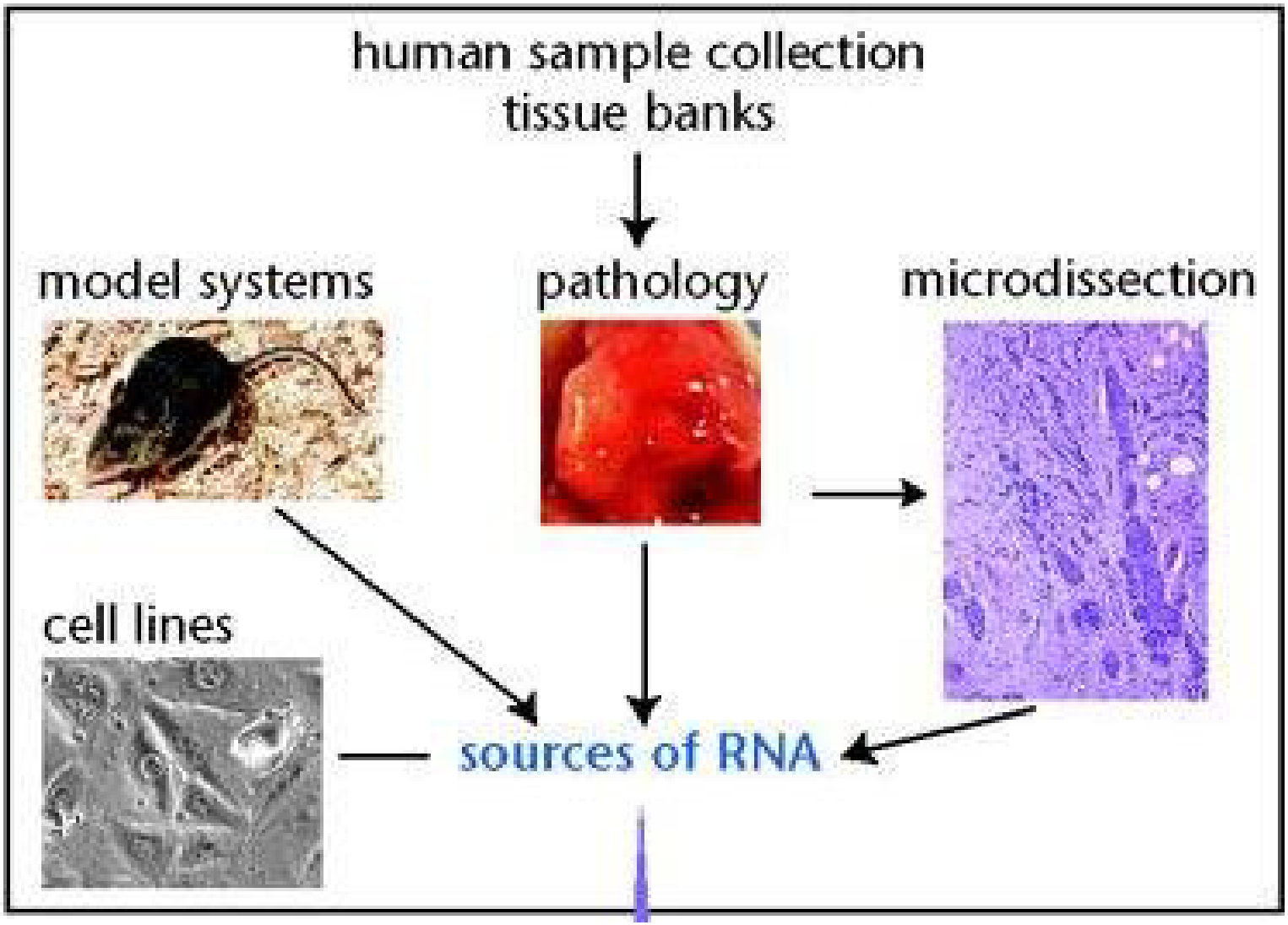
Clustering classification
Network discovery
Integration in to a biological systems
Alternative technology confirmation

MicroArrays Flow-Chart



- RNA extraction
- RNA quality check
- Labeling strategies
- Hybridization
- Image Acquisition
- Data Analysis

Sample Preparation and QC



RNA Extraction method

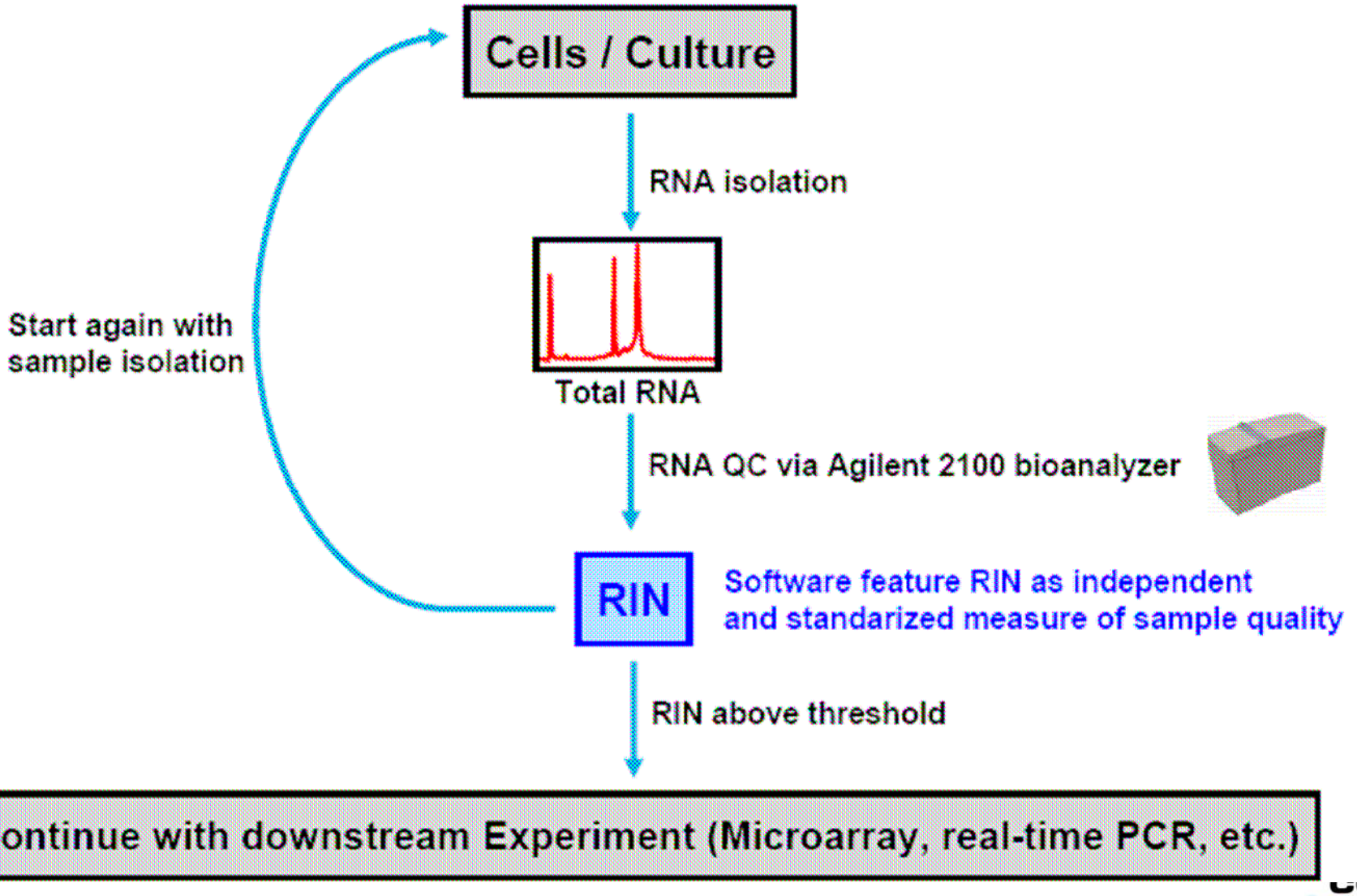
- Use an extraction method specific for your samples
  - Every tissue is different
- Never change protocols during an experiment
  - Every Bias will be translated into Data
- Go for the best quality RNA
  - Degraded RNA turns into Degraded Data

RNA purity measured by light absorbance (OD)

- 260 nm → Nucleic Acids
- 280 nm → Proteins
- 230 nm → other contaminants

- Good quality
  - 260/280 ratio: 1.8-2.2
  - 260/230 ratio: 1.8-2.2

RNA QC in Routine Gene Expression Workflow



Features of the RNA 6000 Assays

total RNA

determine integrity and quality of total RNA

determination of RNA concentration

identify ribosomal peaks

calculate the ratio of ribosomal peaks (18S/28S or 16S/23S)

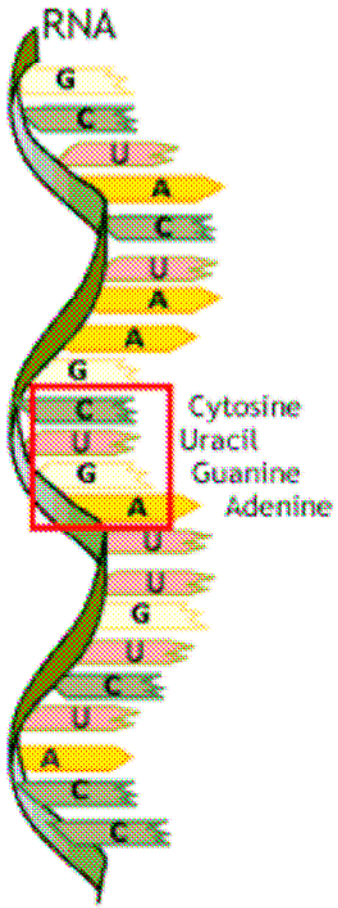
RNA integrity number (RIN)

mRNA

determine integrity and quality of mRNA samples

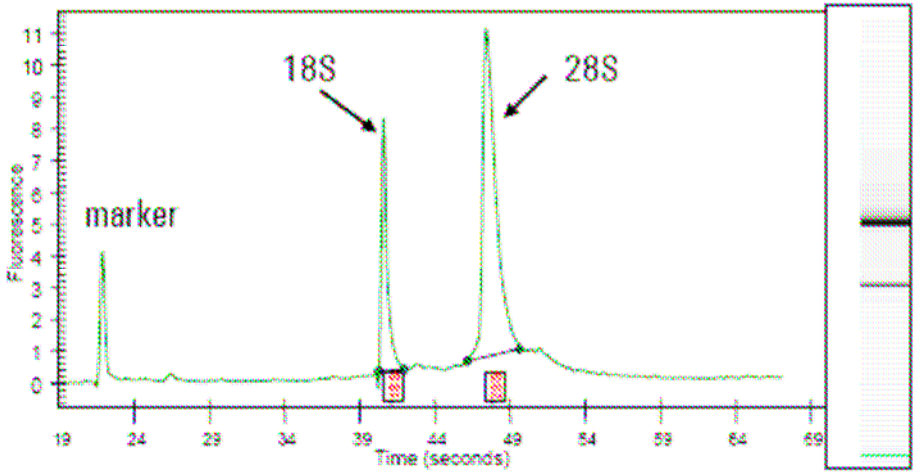
Determination of mRNA concentration

calculate % ribosomal RNA in mRNA samples



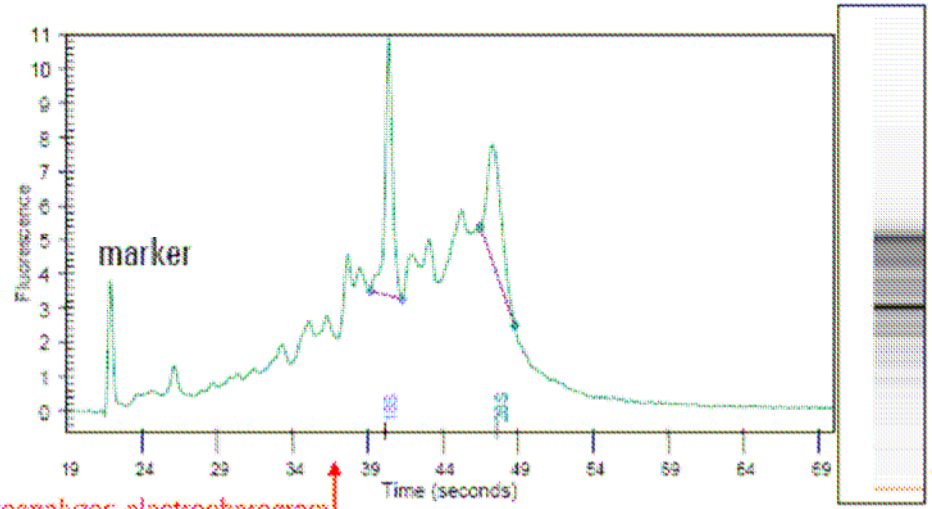
RNA 6000 Nano LabChip kit

Analysis of Total RNA Integrity



Typical first QC step after RNA sample prep prior to microarrays or real-time PCR

High quality total RNA

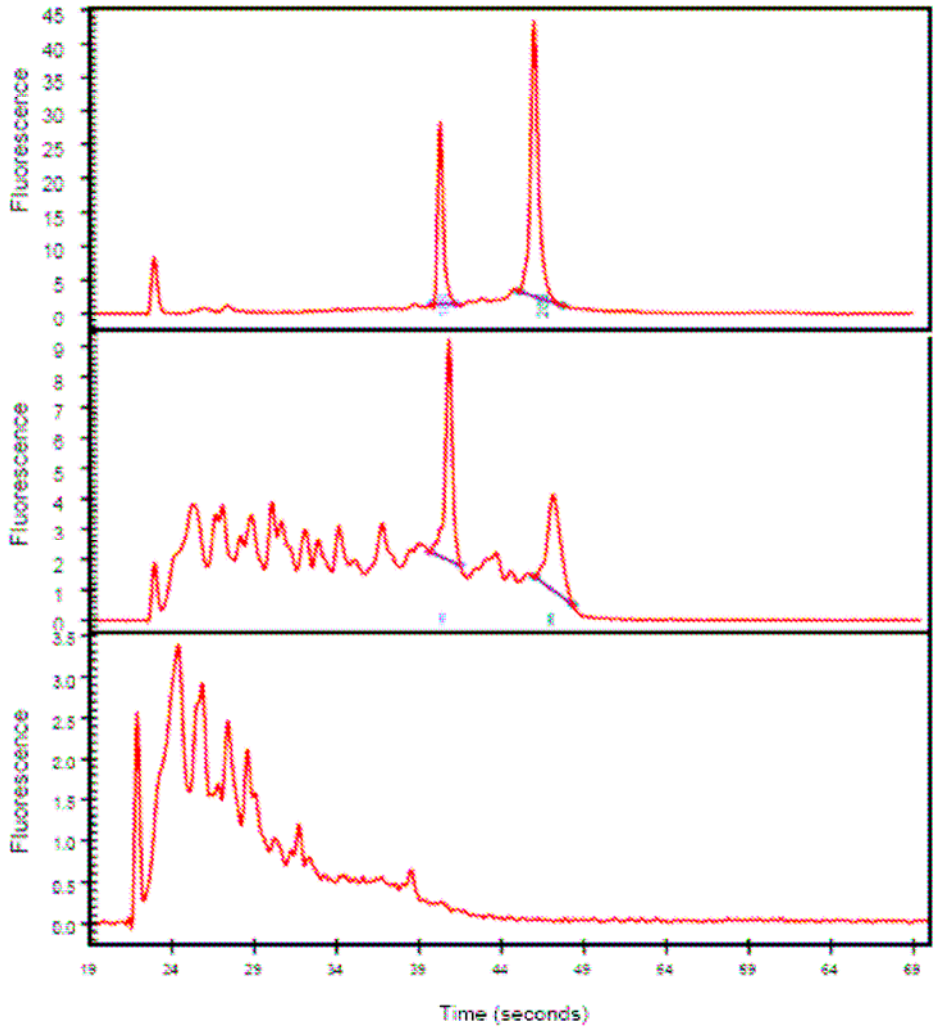


Partially degraded total RNA

2100 bioanalyzer: electropherogram

2100 bioanalyzer: single lane gel-like image

RIN Application – Assessment of RNA Integrity

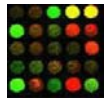


Intact RNA: RIN 10

Partially degraded RNA: RIN 5

Strongly Degraded RNA: RIN 3

Labelling Nucleic Acid



Reverse transcription and Amino-allyl Coupling of RNA

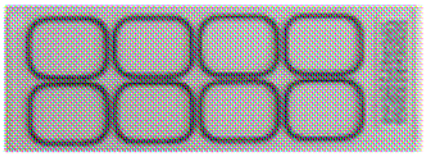
Preparation of Fluorescent cDNA Probe from Human mRNA (alternate protocol)

Modified Eberwine ("ANTISENSE") RNA Amplification Protocol

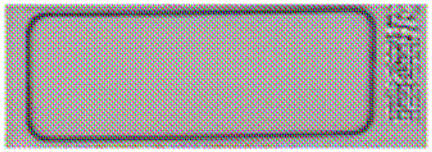
Round A/B DNA Ampification Protocol

Hybridization

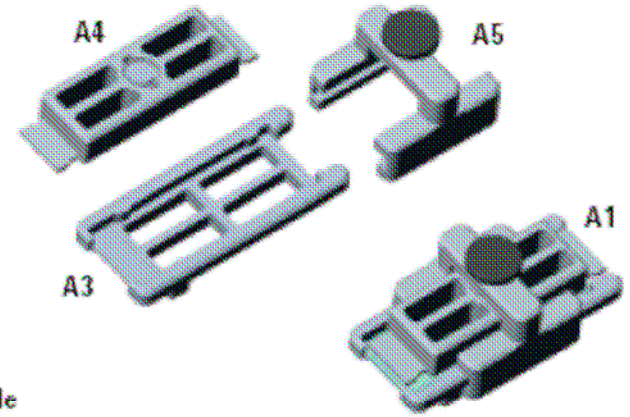
Hybridization ovens



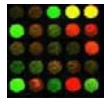
A2a- Experimental 8-well gasketed backing slide



A2b- Single well gasketed backing slide



The Agilent Microarray Scanner.



Feature Extraction software with built-in error models

Flexible 1" x 3" (25mm x 75mm) glass format

48-position slide carousel for walk-away ease

SureScan technology for higher sensitivity scanning with minimal interaction

Peace-of-mind operation backed by Agilent's worldwide service and support

Tight resolution (5 micron) scanning and data acquisition

Dual-laser scanning for 2-color microarray formats

Rugged optics and optical bench for optimum scans

Gene expression analysis of KCL22 cell lines for the identification of molecular mechanisms associated to imatinib resistance

Analysis of 22.000 human genes using 60-mer oligonucleotides (Agilent) sinthetized on glass slides

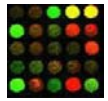
Cell lines comparison and Time Course

For each experiment swap of dyes

Image acquisition with the Agilent Scanner

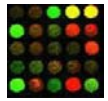
Data Analysis performed with GeneSpring, Babelomics and GO Tree Machine

Chronic myeloid leukemia



Chronic myeloid leukemia (CML) is a clonal disorder of the pluripotent hemopoietic stem cell, in which a reciprocal translocation t(9;22)(q34;q11) forms a Philadelphia (Ph) chromosome and creates a novel fusion gene, bcr–abl. Its corresponding protein has a constitutively activated tyrosine kinase that is central to the pathogenesis of CML.

Imatinib clinical effect on chronic myelogenous leukemia



Imatinib **(formally STI571)** has been reported to have a significant clinical effect on chronic myelogenous leukemia (CML) in blast crisis as well as in the chronic phase

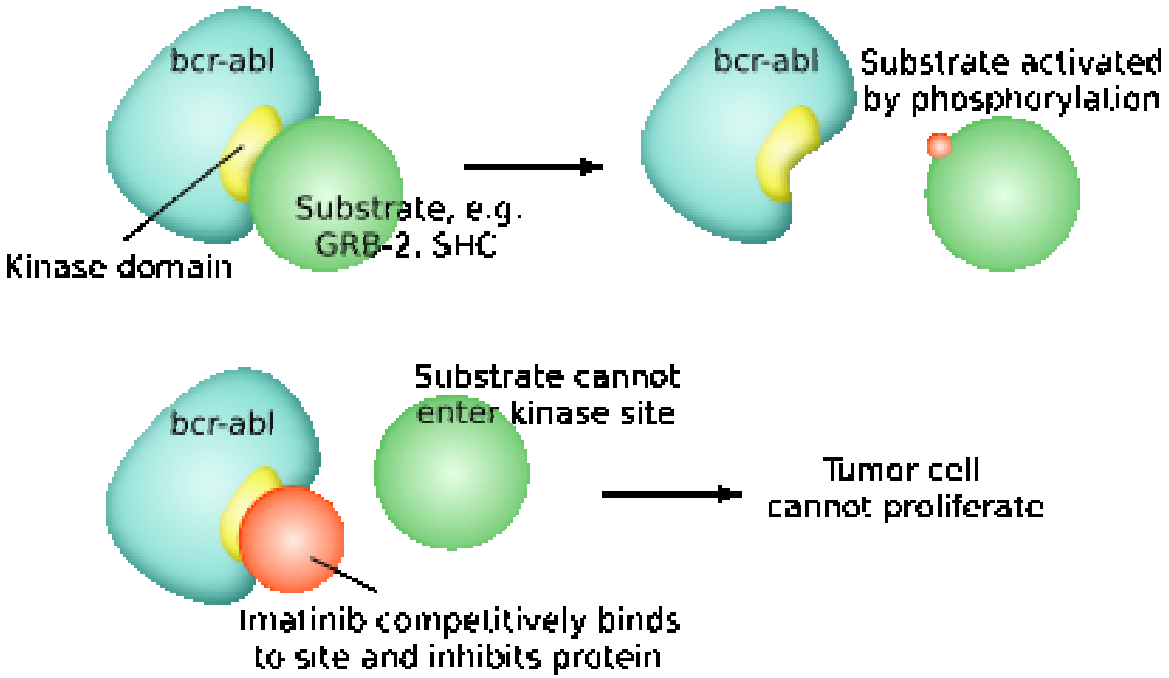
Imatinib mesylate is a selective BCR-ABL protein tyrosine kinase inhibitor against:

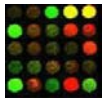
ABL oncoproteins (c-ABL, BCR-ABL, ETV1-ABL),

c-KIT

Platelet-derived growth factor receptor

Mechanism of the drug imatinib

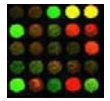




Many patients in blast crisis who are being treated with imatinib relapse at a relatively early time, suggesting that leukemia cells tend to acquire resistance to imatinib easily in blast crisis.

Drug resistance is a major problem for CML patients in blast crisis who are being treated with imatinib.

Imatinib resistance mechanisms



Intrinsic resistance to imatinib

src-kinase LYN

Constitutive nuclear factor (NF)-B activation

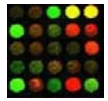
Secondary or acquired resistance

Mutations in the BCR–ABL domain

Overexpression of BCR–ABL oncoprotein

Neither overexpression nor mutations of BCR/ABL have been found in some imatinib-resistant cell lines and patients

KCL22 and KCL22R Cell Lines



KCL22 is a Philadelphia chromosome-positive cell line established from peripheral blood of a patient with CML in blast crisis

To generate imatinib-resistant clones, KCL22 cells were treated with step-wise increasing concentrations of imatinib (0.1–1.0 μM)

The IC50 value of imatinib for KCL22/SR is about 11.6-fold higher than that to KCL22 indicating a significant resistance to imatinib

No mutation in the BCR/ABL gene and no increase in BCR/ABL protein and P-gp levels were observed

Gene expression analysis of KCL22 cell lines for the identification of molecular mechanisms associated to imatinib resistance

Analysis of 22.000 human genes using 60-mer oligonucleotides (Agilent) sinthetized on glass slides

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For each experiment swap of dyes

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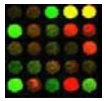
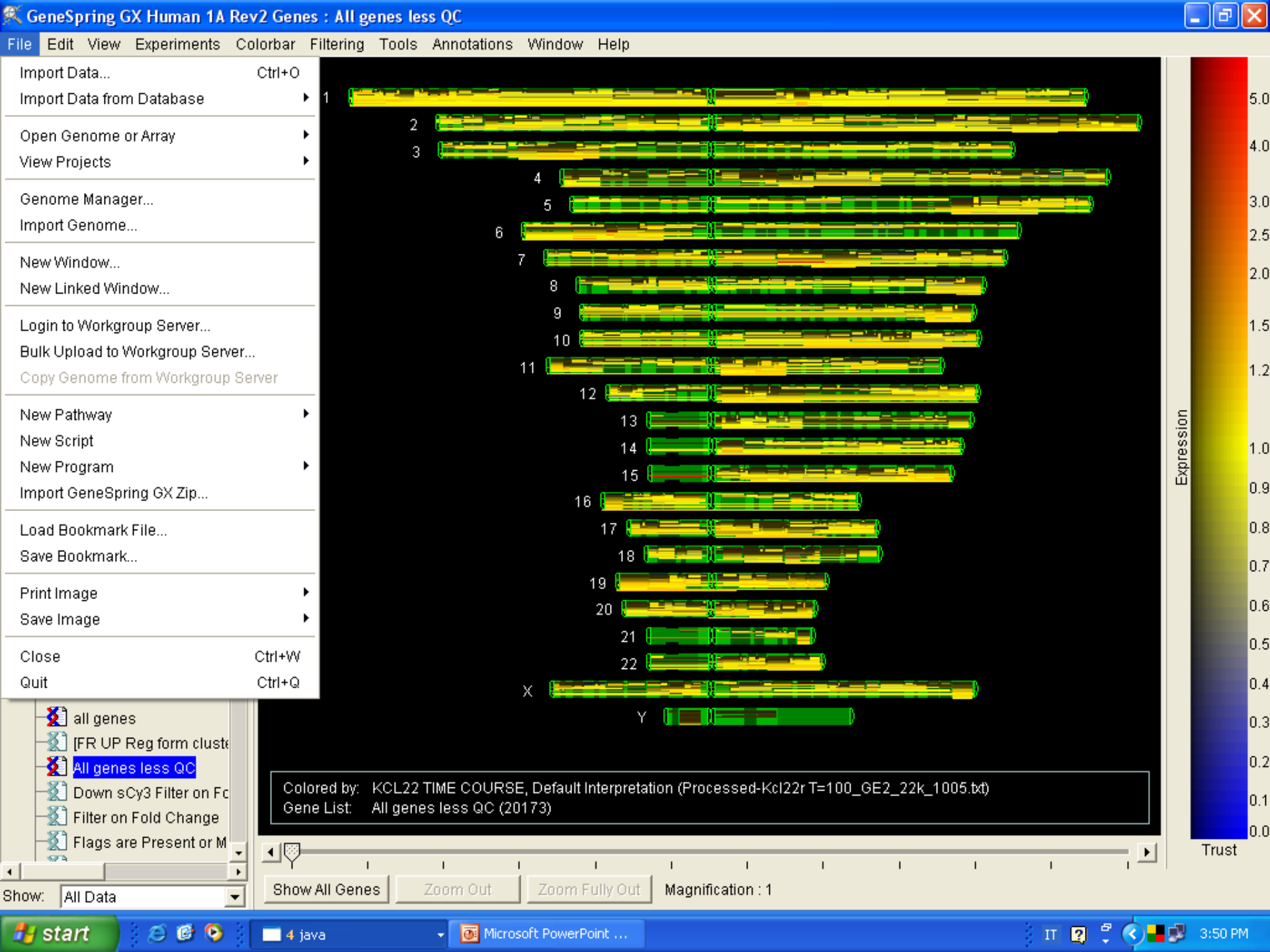


GeneSpring Data Analysis

Rosanna Martinelli Ph.D. Dipartimento di Biochimica e Biotecnologie Mediche - CEINGE Biotecnologie Avanzate


GeneSpring GX Human 1A Rev2 Genes : All genes less QC
File Edit View Experiments Colorbar Filtering Tools Annotations Window Help
Import Data... Ctrl+O
Import Data from Database
Open Genome or Array
View Projects
Genome Manager...
Import Genome...
New Window...
New Linked Window...
Login to Workgroup Server...
Bulk Upload to Workgroup Server...
Copy Genome from Workgroup Server
New Pathway
New Script
New Program
Import GeneSpring GX Zip...
Load Bookmark File...
Save Bookmark...
Print Image
Save Image
Close Ctrl+W
Quit Ctrl+Q
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y
Expression
5.0 4.0 3.0 2.5 2.0 1.5 1.2 1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
Trust
all genes
[FR UP Reg form cluste
All genes less QC
Down sCy3 Filter on Fc
Filter on Fold Change
Flags are Present or M
Colored by: KCL22 TIME COURSE, Default Interpretation (Processed-Kcl22r T=100_GE2_22k_1005.txt)
Gene List: All genes less QC (20173)
Show: All Data
Show All Genes
Zoom Out
Zoom Fully Out
Magnification : 1
start
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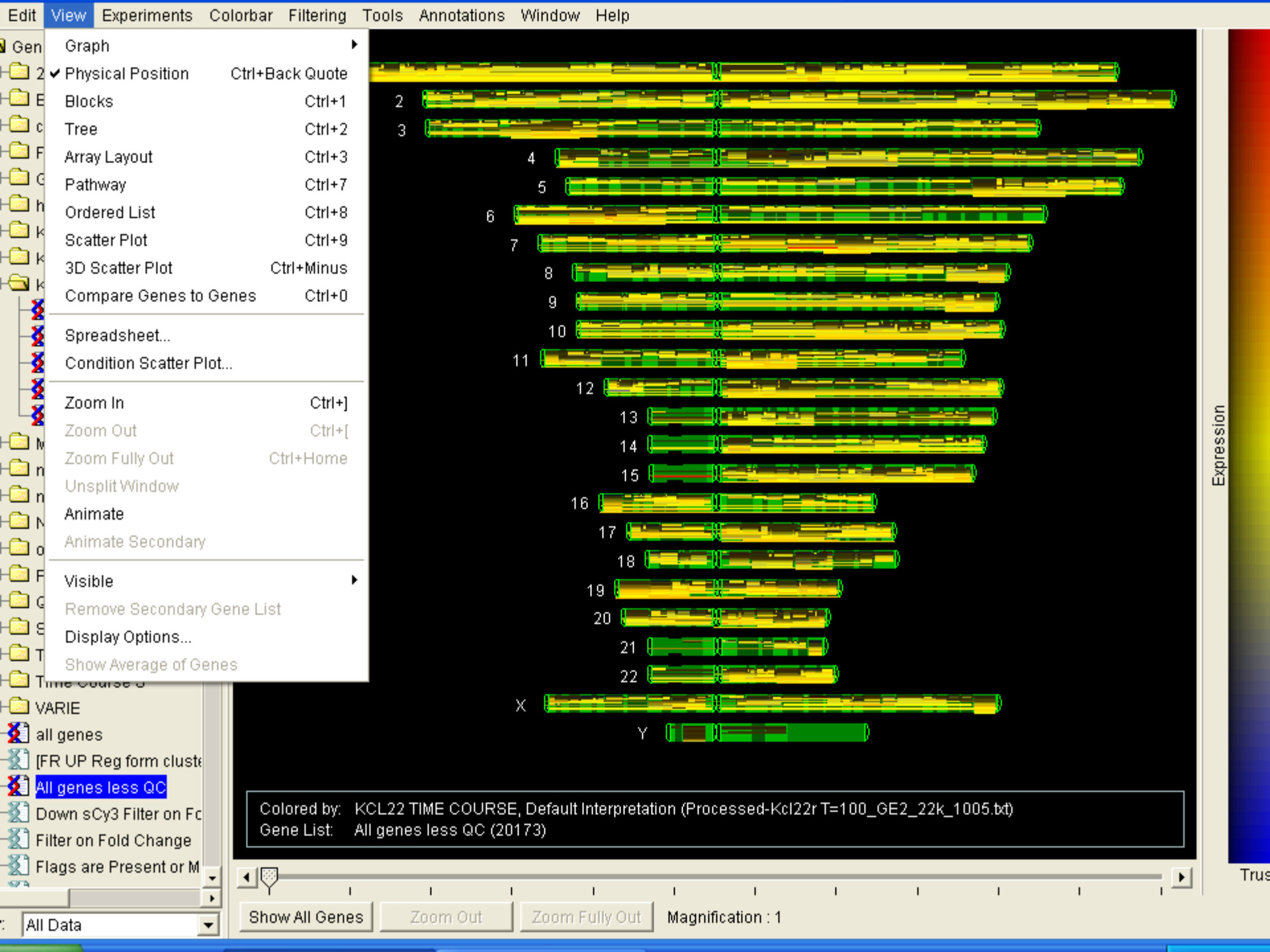
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Tree Ctrl+2
Array Layout Ctrl+3
Pathway Ctrl+7
Ordered List Ctrl+8
Scatter Plot Ctrl+9
3D Scatter Plot Ctrl+Minus
Compare Genes to Genes Ctrl+0
Spreadsheet...
Condition Scatter Plot...
Zoom In Ctrl+]
Zoom Out Ctrl+[
Zoom Fully Out Ctrl+Home
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Animate
Animate Secondary
Visible
Remove Secondary Gene List
Display Options...
Show Average of Genes
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Colored by: KCL22 TIME COURSE, Default Interpretation (Processed-Kcl22r T=100_GE2_22k_1005.txt)
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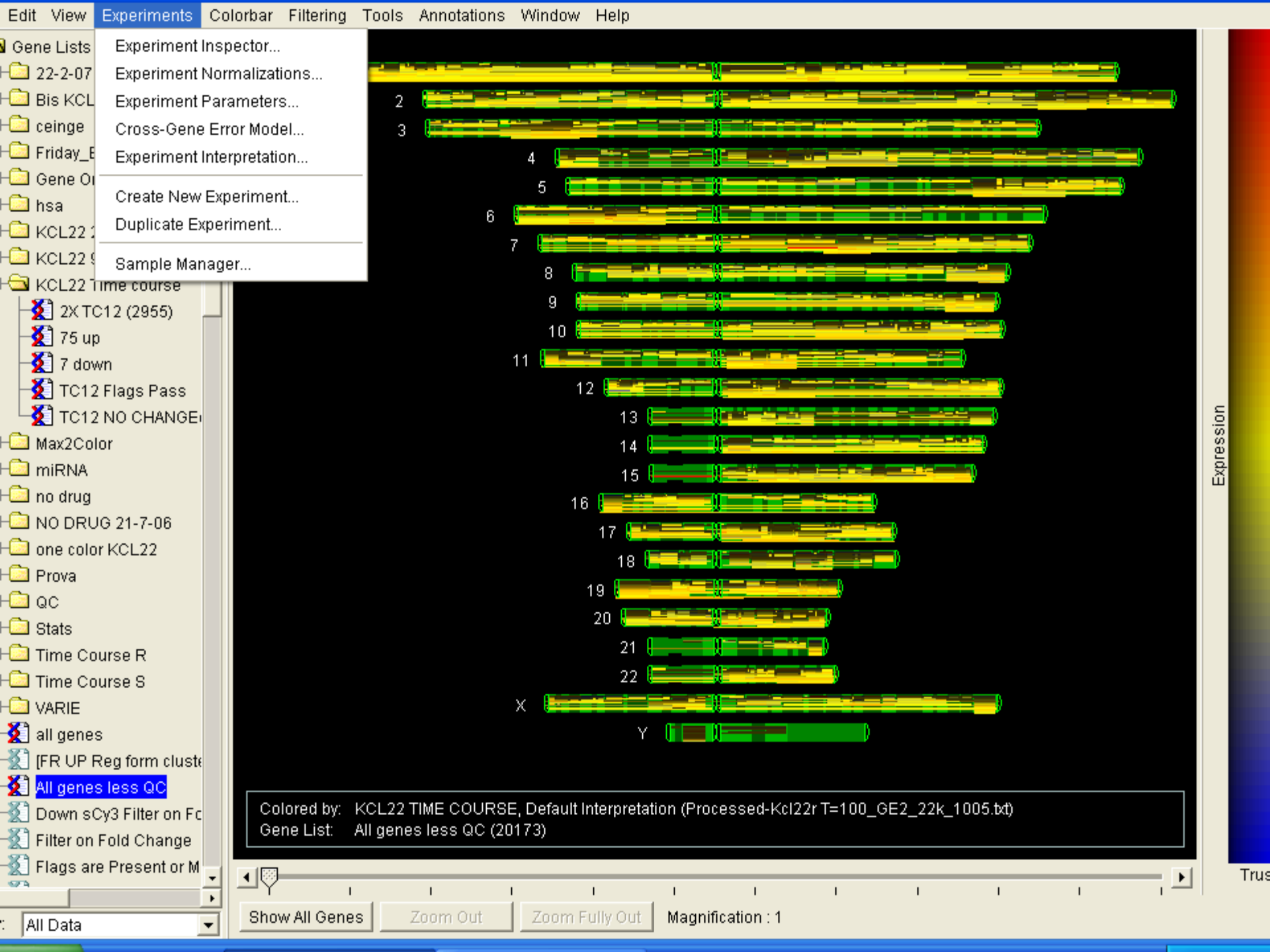
Edit View Experiments Colorbar Filtering Tools Annotations Window Help
Experiment Inspector...
Experiment Normalizations...
Experiment Parameters...
Cross-Gene Error Model...
Experiment Interpretation...
Create New Experiment...
Duplicate Experiment...
Sample Manager...
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Gene List: All genes less QC (20173)
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Show All Genes
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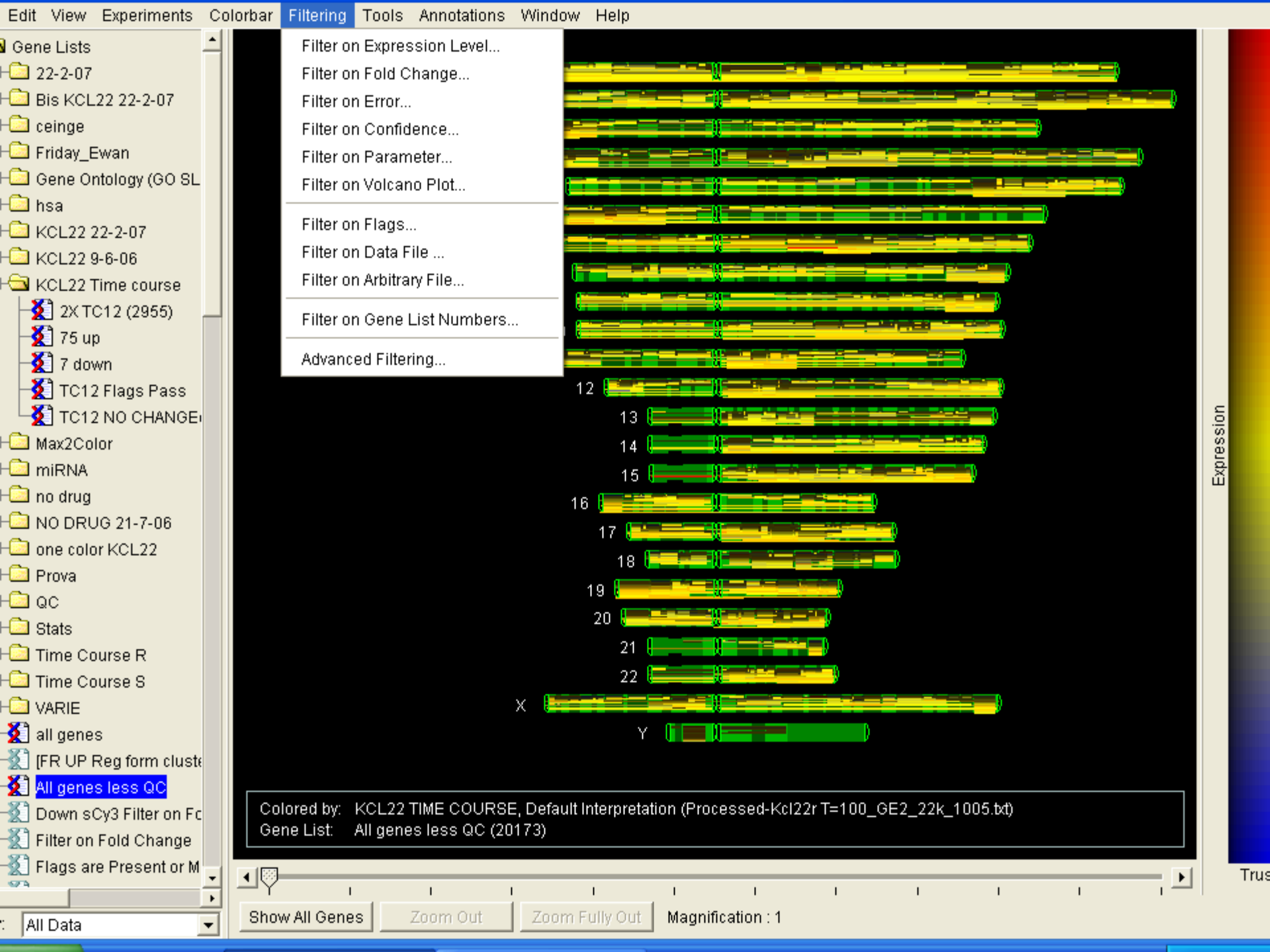
Edit View Experiments Colorbar Filtering Tools Annotations Window Help
Filter on Expression Level...
Filter on Fold Change...
Filter on Error...
Filter on Confidence...
Filter on Parameter...
Filter on Volcano Plot...
Filter on Flags...
Filter on Data File ...
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Filter on Gene List Numbers...
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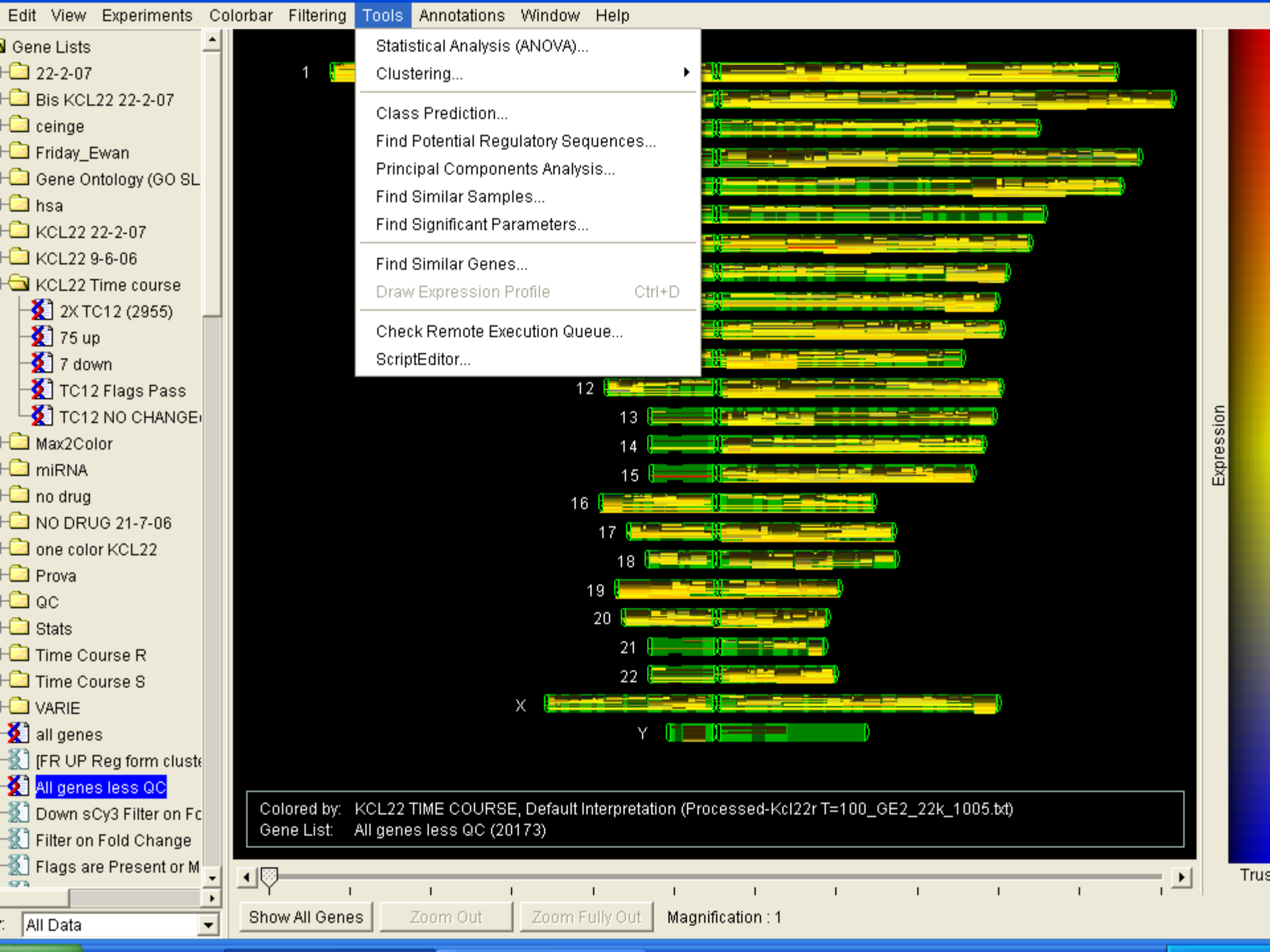
Edit View Experiments Colorbar Filtering Tools Annotations Window Help
Statistical Analysis (ANOVA)...
Clustering...
Class Prediction...
Find Potential Regulatory Sequences...
Principal Components Analysis...
Find Similar Samples...
Find Significant Parameters...
Find Similar Genes...
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Gene List: All genes less QC (20173)
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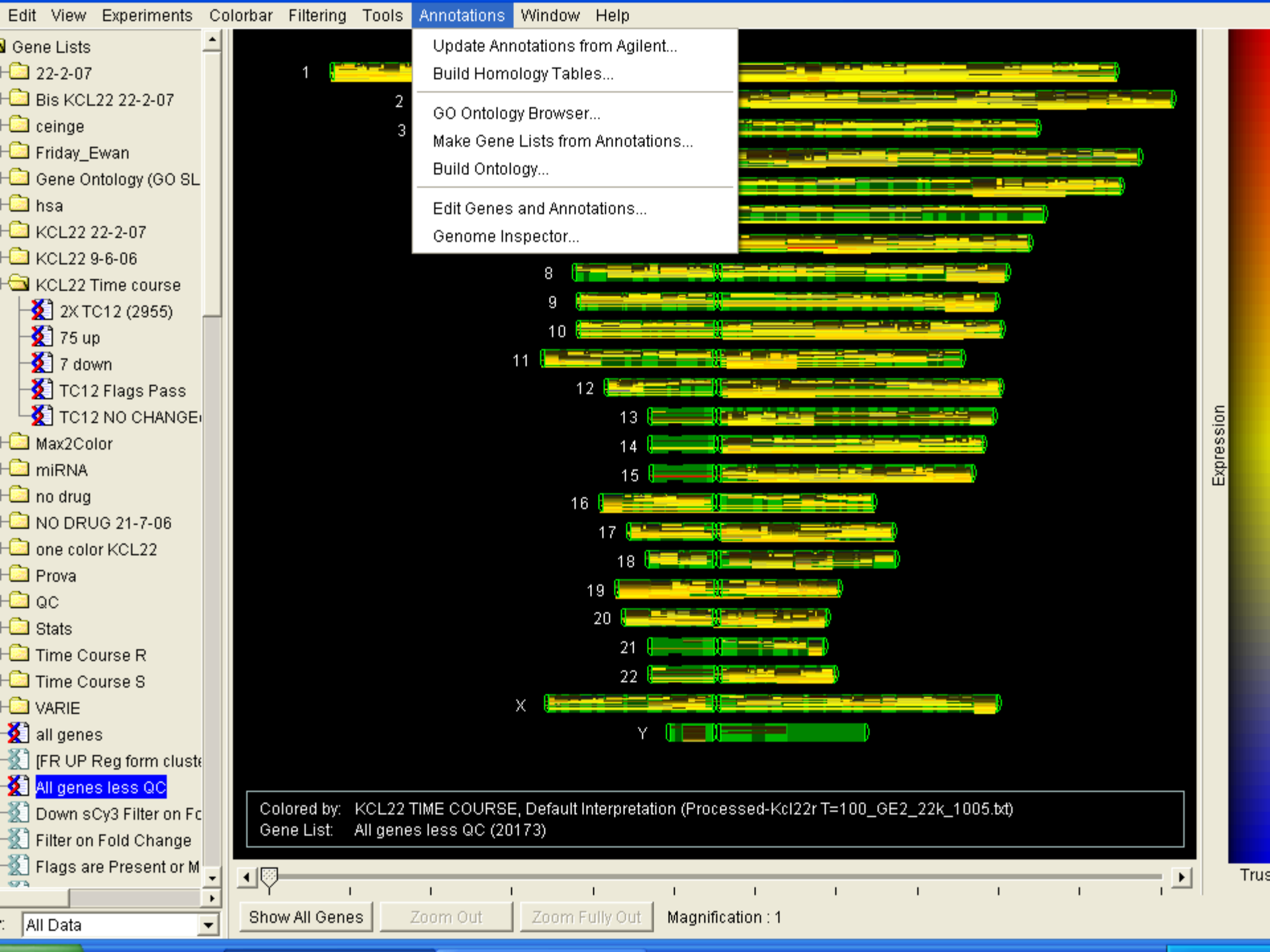
Edit View Experiments Colorbar Filtering Tools Annotations Window Help
Update Annotations from Agilent...
Build Homology Tables...
GO Ontology Browser...
Make Gene Lists from Annotations...
Build Ontology...
Edit Genes and Annotations...
Genome Inspector...
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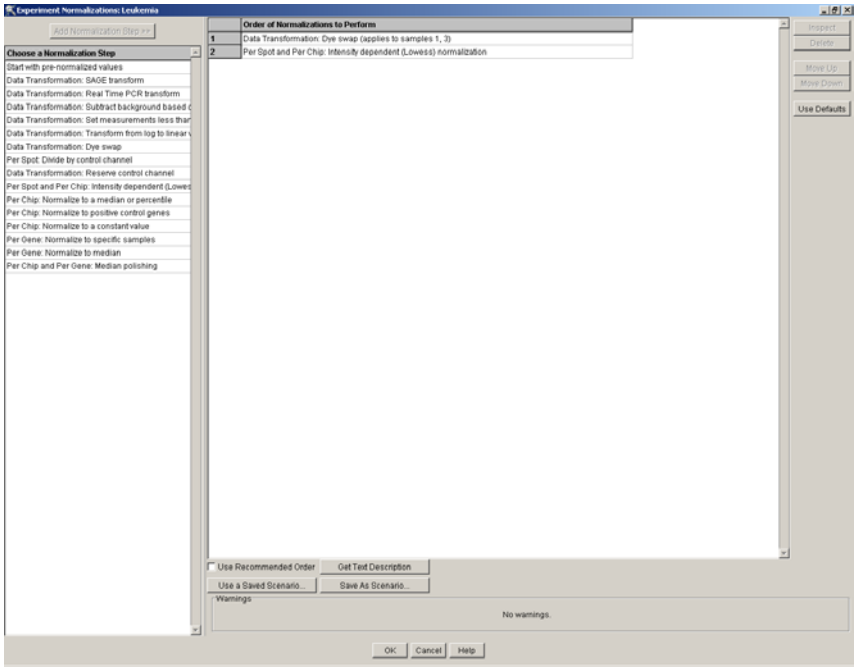
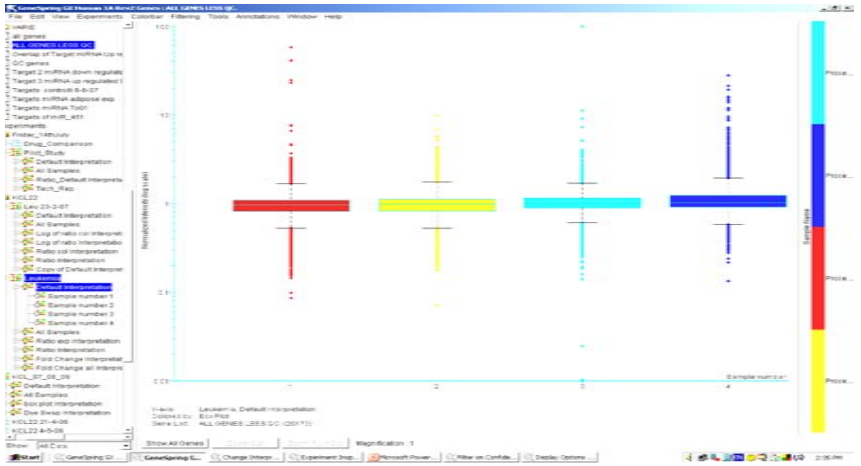
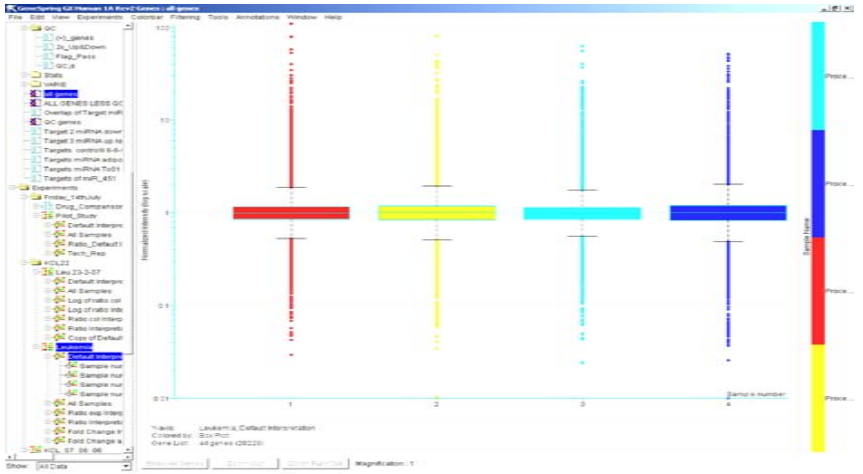
Expression
Colored by: KCL22 TIME COURSE, Default Interpretation (Processed-Kcl22r T=100_GE2_22k_1005.txt)
Gene List: All genes less QC (20173)
Show All Genes
Zoom Out
Zoom Fully Out
Magnification : 1

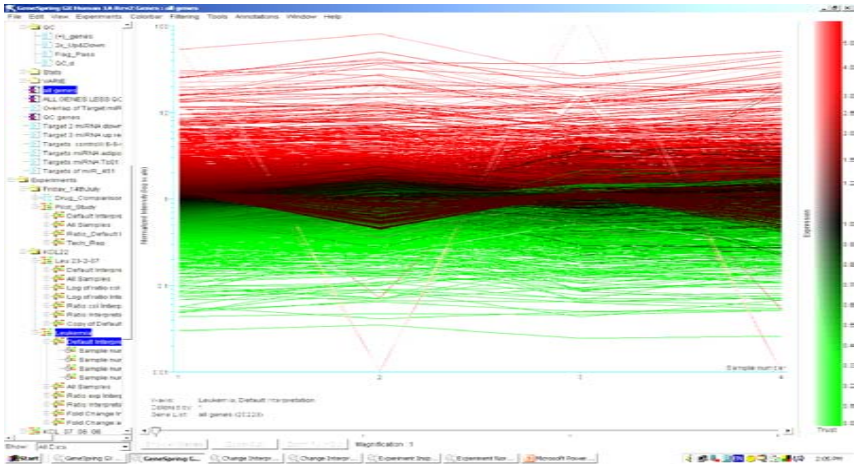
GeneSpring data normalization

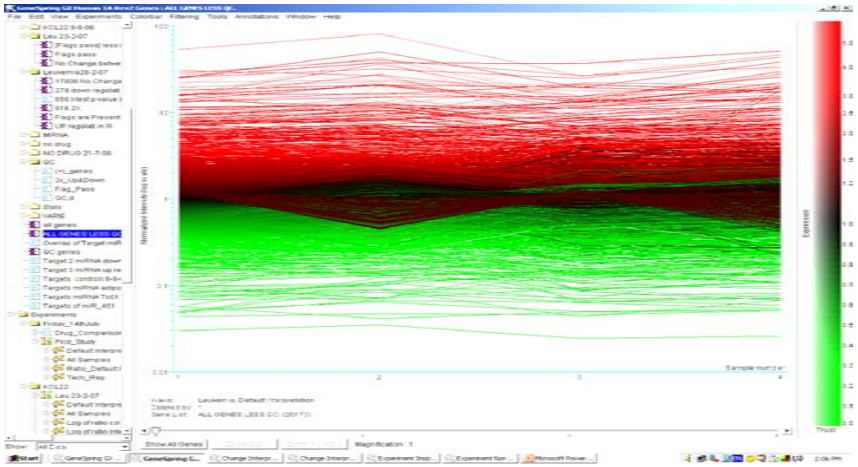


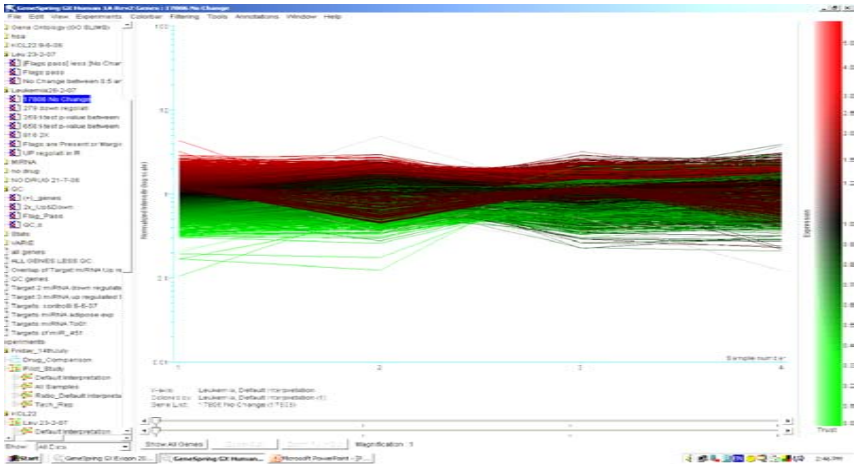


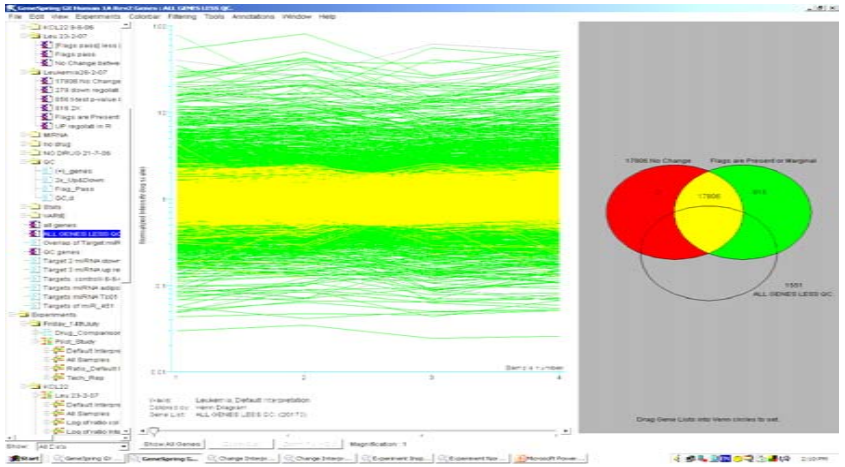


GeneSpring Data Processing

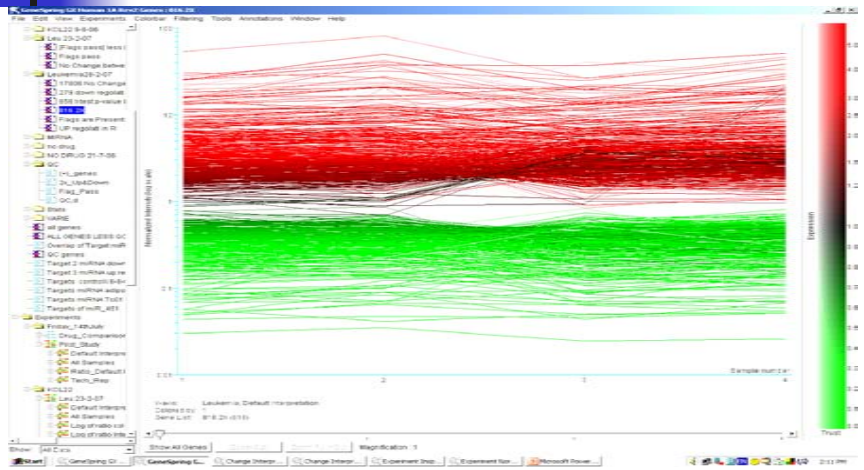


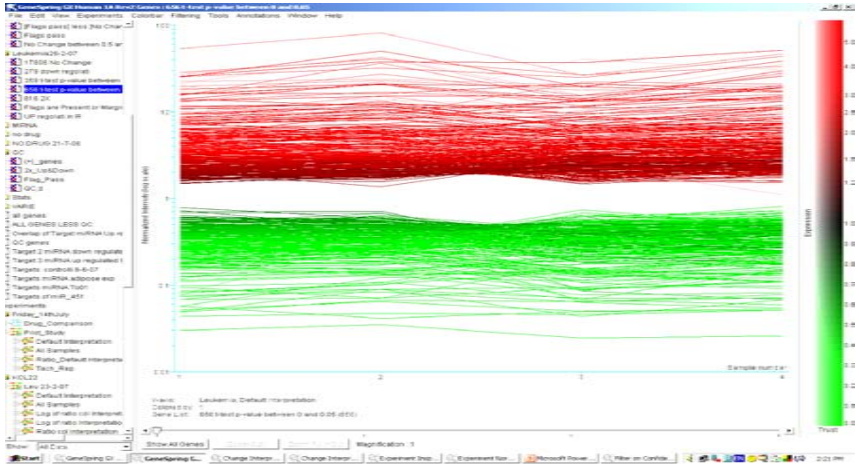


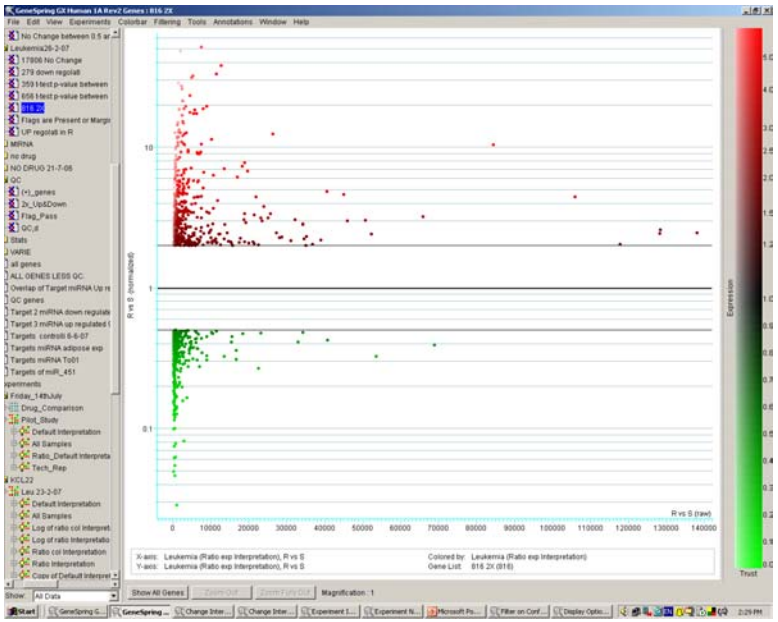




GeneSpring Statistical Analysis

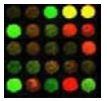






Rosanna Martinelli Ph.D. Dipartimento di Biochimica e Biotecnologie Mediche - CEINGE Biotecnologie Avanzate

Gene Sorting & Interpretation



Gene Ontology

the GO project has developed three structured controlled vocabularies (ontologies) that describe gene products in terms of their associated

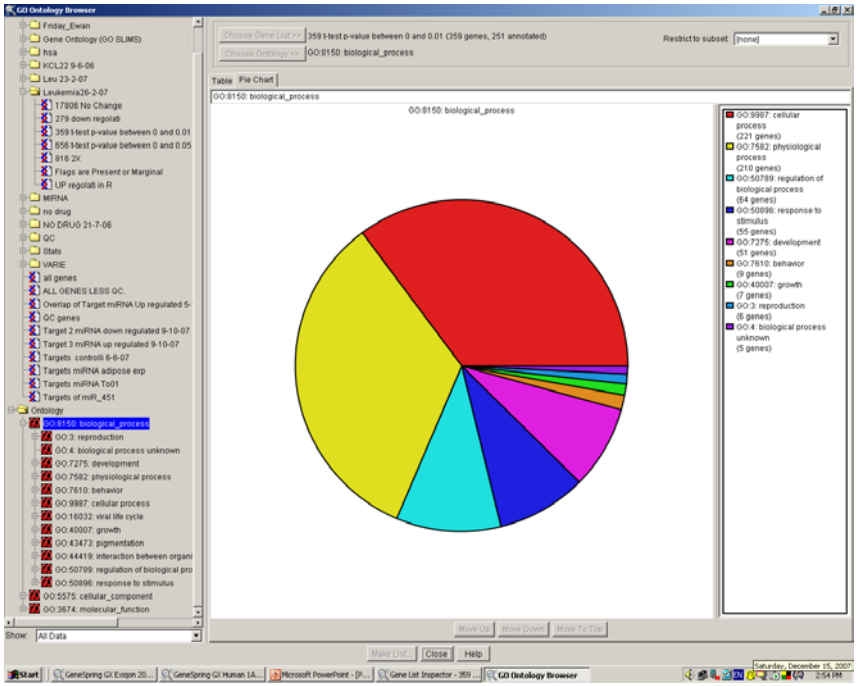
- Biological Process
- Molecular Function
- Cellular Pathways

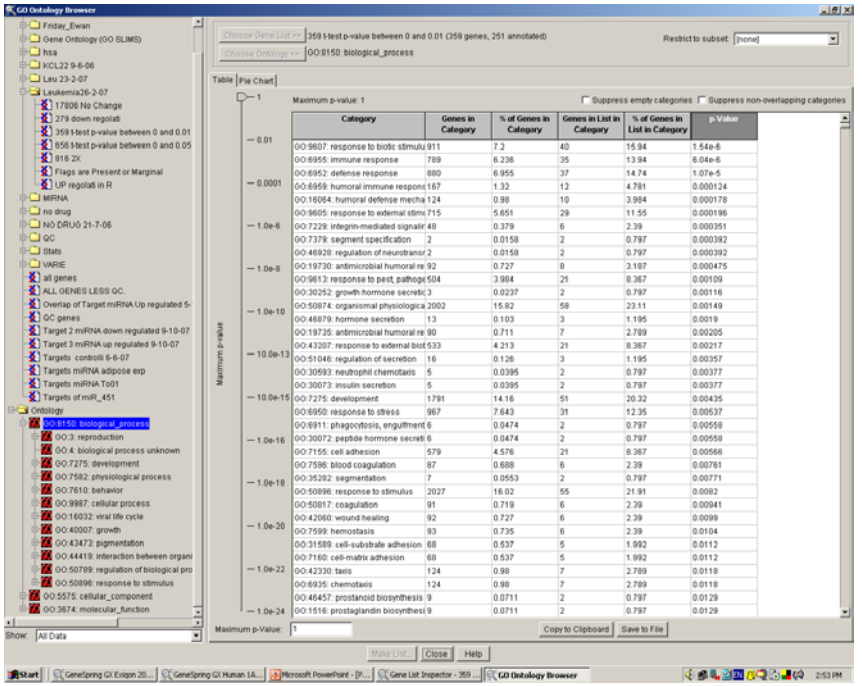
KEGG Pathways

is a collection of manually drawn pathway maps representing knowledge on the molecular interaction and reaction networks for:

- Metabolism
- Genetic information
- Enviromental Information
- Cellular Process
- Human diseases
- Drug Development

Gene Ontology





Edit View Experiments Colorbar Filtering Tools Annotations Window Help

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  - Time Course
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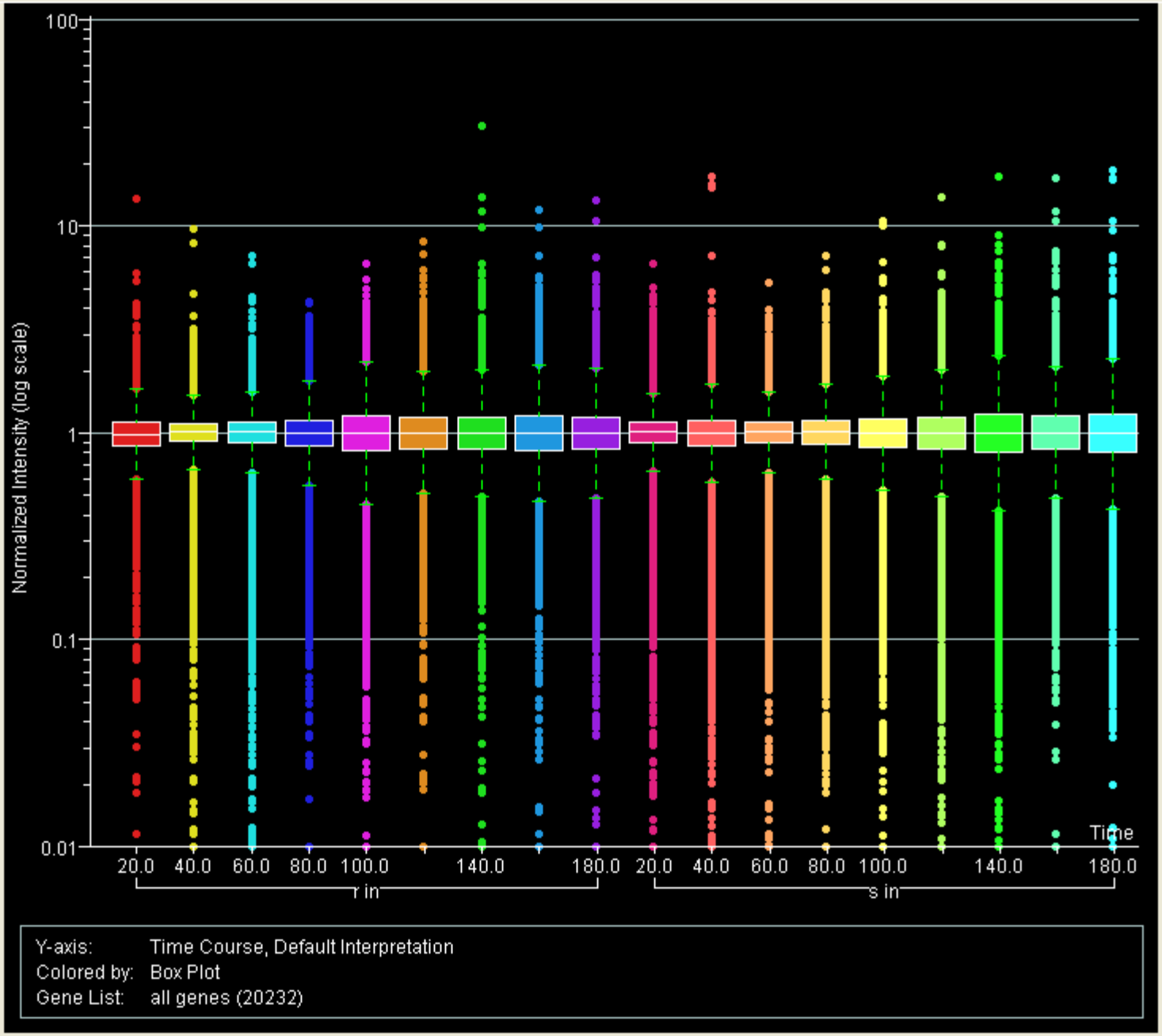
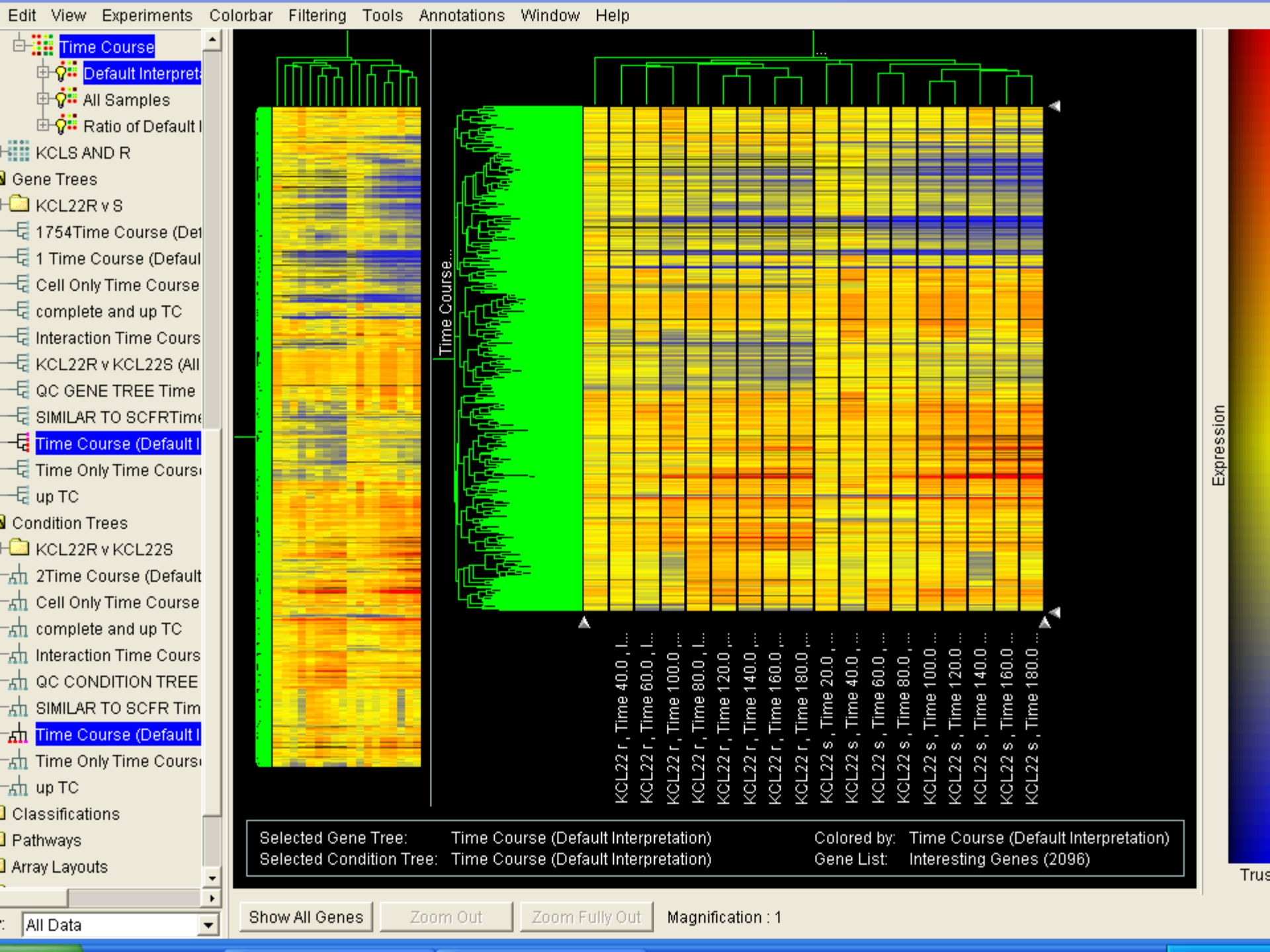
      - Default Interpret
      - All Samples
      - Ratio of Default I
  - KCLS AND R
- Gene Trees
- Condition Trees
- Classifications
- Pathways
- Array Layouts
- Expression Profiles
- External Programs

All Data



Show All Genes | Zoom Out | Zoom Fully Out | Magnification : 1


Edit View Experiments Colorbar Filtering Tools Annotations Window Help
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Selected Condition Tree: Time Course (Default Interpretation)
Colored by: Time Course (Default Interpretation)
Gene List: Interesting Genes (2096)
Show All Genes
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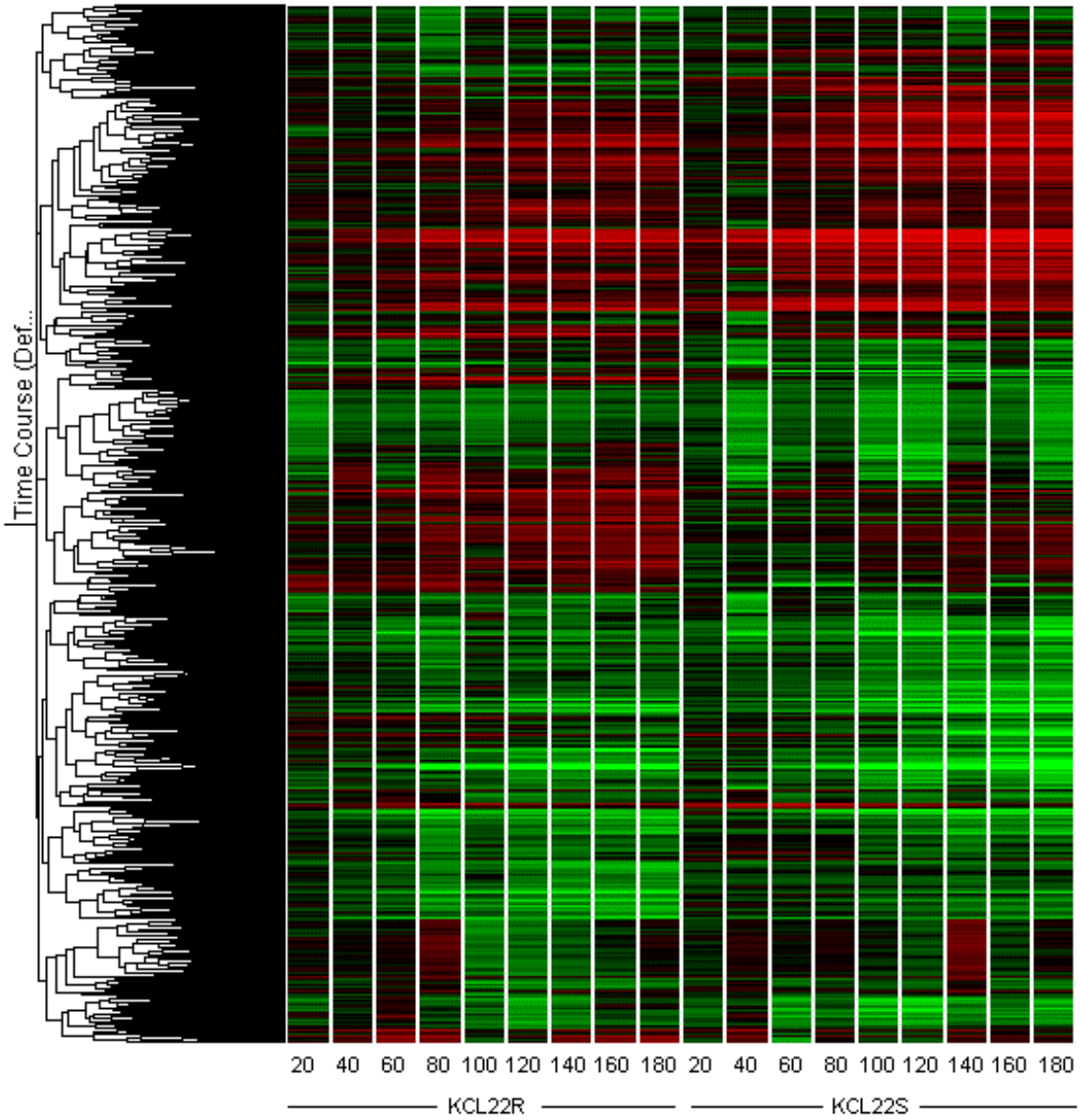
Zoom Fully Out
Magnification : 1

KCL22 Time Course



Selected Gene Tree:	Time Course (Default Interpretation)	Colored by:	Time Course (Default Interpretation)
Selected Condition Tree:	Time Course (Default Interpretation)	Gene List:	2nd interesting (644)

Acknowledgements

Dott.ssa Tonia Buonomo
Dott.ssa Vera Roberti
Prof. Fabrizio Pane
Sig. Vittorio Lucignano

Prof. Francesco Salvatore