FLOW CYTOMETRIC ANALYSIS OF CELLS FOR DNA: AN OVERVIEW

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THE PLAN

DNA ANALYSIS BY FLOW CYTOMETRY:

• WHEN (and WHO)?
  • HOW?
  • WHAT?

DNA analysis teaches us about the DNA content of cells but also forces us to learn how to use a flow cytometer correctly.
WHEN (and WHO) ?

• 1965: KAMENTSKY : Pap smear by 260 nm absorption
• 1969: VAN DILLA : DNA histogram
• 1969: GÖHDE : ethidium bromide
• 1973: CRISSMAN : propidium iodide
• 1974: DEAN and JETT : cell cycle analysis
• 1975: KRISHAN : permeabilizing cells
• 1975: GRAY : chromosomes
• 1976: LATT : Hoechst dyes
• 1980: DARZYNKIEWICZ : with RNA
• 1981: DAVIES : gene library from sorted chromosomes
• 1983: HEDLEY : paraffin-embedded material
• 1985: CLEVENGER : DNA with immunofluorescence
• 1989: JETT : base sequencing
• 1991: MANY : apoptosis
• 1993: MANY : cyclins and cell cycle regulation
• 1996 to present : Los Alamos : DNA fragment sizing
KAMENTSKY, MELAMED, AND DERMAN: “NEW INSTRUMENT FOR ULTRARAPID CELL ANALYSIS”

Kamentsky, Melamed, and Derman, Science 150: 630-631, 1965
PAP SMEAR BY FLOW CYTOMETRY: 1965

410nm BACK SCATTER

Kamentsky, Melamed, and Derman, Science 150: 630-631, 1965
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DNA ANALYSIS BY FLOW CYTOMETRY:

• WHEN (and WHO) ?
• HOW?
  • CELLS
  • FLUOROCHROMES
• WHAT ?
DNA in nucleus fluoresces brightly; double-stranded RNA in the cytoplasm also fluoresces.

DNA fluorochromes can be compatible with cytoplasmic and surface staining.
HEADS UP ON DNA ANALYSIS

• PREPARATION OF CELLS
• ACCESSIBILITY OF FLUOROCHROMES TO DNA
• CHARACTERISTICS OF FLUOROCHROMES
• ALIGNMENT OF PARTICLES IN LASER BEAM
• APPLICATION OF MATH TO BIOLOGY
• AGGREGATES
HOW (CELL PREPARATIONS)?

- LIVE CELLS?  OR
- FREE NUCLEI
- FIXED CELLS
- PERMEABILIZED CELLS
- FIXED AND PERMEABILIZED CELLS
- CHROMOSOMES
- SPERM
- DNA FRAGMENTS
FIXATION and
PERMEABILIZATION
CRITERIA IN STAINING FOR DNA WITH A SECOND PARAMETER

- DNA ACCESSIBLE TO FLUOROCHROMES
- PRESERVATION OF OUTER MEMBRANE
- PRESERVATION OF CYTOPLASM
- ANTIGENS UNCHANGED BY FIXATION

LIFE (AND FLOW CYTOMETRY) REQUIRE COMPROMISES
HEADS UP ON DNA ANALYSIS

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• AGGREGATES
DNA STRUCTURE
CHROMATIN SUPERSTRUCTURE

PROTEIN CROSS-LINKING BY FORMALDEHYDE
see French and Edsall, Advances in Protein Chemistry, 1945
HEADS UP ON DNA ANALYSIS

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- APPLICATION OF MATH TO BIOLOGY
- CLUMPS
KNOW THE SPECS OF YOUR FLUOROCHROMES

USE WEB SITES, BOOKS, AND CATALOGS*

<table>
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<th>PROBE</th>
<th>ABS</th>
<th>EM</th>
<th>$\varepsilon \times 10^{-3}$</th>
<th>QY</th>
<th>$F_{\text{bound}}/F_{\text{free}}$</th>
<th>TARGET</th>
<th>BASE</th>
<th>PERMEANT?</th>
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<td>460</td>
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<td>AT</td>
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<td>~20</td>
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<td>6</td>
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<tr>
<td>ToTo-3</td>
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* these values have been compiled from Waggoner, in Flow Cytometry and Sorting (Melamed et al, eds), from Haugland, Molecular Probes Handbook, and from Poot, in Current Protocols in Cytometry (Robinson et al, eds)
MOLECULAR PROBES FLUOROCHROMES

\[ X = S \text{ for TOPRO dyes} \quad n = 0 \text{ for } \cdots 1 \]
\[ X = O \text{ for YOPRO dyes} \quad n = 1 \text{ for } \cdots 3 \]

structure is a mirrored dimer
for TOTO and YOYO
HEADS UP ON DNA ANALYSIS

• PREPARATION OF CELLS
• ACCESSIBILITY OF FLUOROCHROMES TO DNA
• CHARACTERISTICS OF FLUOROCHROMES
• ALIGNMENT OF PARTICLES IN LASER BEAM
• APPLICATION OF MATH TO BIOLOGY
• CLUMPS
A NARROW CORE HELPS TO MAINTAIN CELLS IN THE BRIGHT PART OF THE LASER BEAM AND HELPS TO AVOID COINCIDENCE OF TWO CELLS IN THE LASER BEAM AT THE SAME TIME
CELLS FLOWING THROUGH A LASER BEAM: with a wide core, the cells are not equally illuminated and multiple cells may coincide in the laser beam.
IMMUNOFLUORESCENCE

vs

DNA FLUORESCENCE

Compare the range of intensities of cells stained for the CD3 receptor with the range of intensities of cells stained for DNA (this is why DNA analysis puts great demands on the alignment of the cytometer)
core diameter as a function of cell concentration and cell flow rate (stream velocity 10 m/s)

- $10^5$ cells/ml
- $5 \times 10^5$ cells/ml
- $10^6$ cells/ml
- $5 \times 10^6$ cells/ml

cell flow rate (cells/second)
PROBABILITY OF A FLOW CYTOMETRIC “EVENT” HAVING RESULTED FROM TWO CELLS IN THE LASER BEAM AT THE SAME TIME
ONE FOR THE PRICE OF TWO

for a stream velocity of 10 meters per second and a laser beam 30 µm high
HOW TO MAKE DOUBLE POSITIVES LESS RARE: CREATE PSEUDO-AGGREGATES (COINCIDENCE)

File: 130cps.001
Quad % Gated
UL  42.87
UR  0.07
LL  23.13
LR  33.93

File: 75cps.002
Quad % Gated
UL  42.84
UR  0.04
LL  21.92
LR  35.20

File: 800cps.001
Quad % Gated
UL  43.01
UR  0.23
LL  21.23
LR  35.53

File: 1500cps.002
Quad % Gated
UL  42.80
UR  0.59
LL  21.85
LR  34.76

75 cps
130 cps
800 cps
1500 cps

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DNA ANALYSIS BY FLOW CYTOMETRY:

• WHEN (and WHO) ?
• HOW ?
• WHAT ?
WHAT?

• PLOIDY
• CELL CYCLE
• VIABILITY
• APOPTOSIS
• CHROMOSOME KARYOTYPE
• CHROMOSOME MAPPING
• BASE SEQUENCES
• FRAGMENT SIZES
• SORTING SPERM

} not in this lecture
ANEUPLOIDY DETERMINATIONS: SOMETIMES EASY
ANEUPLOIDY DETERMINATIONS: SOMETIMES EASY, SOMETIMES NOT
CELL CYCLE ANALYSIS:

adapted from Alberts et al, 1989

adapted from Gray et al, 1990
Cell Cycle Analysis

DIPLOID: 100.00 %
Dip G0-G1: 54.79 % at 76.28
Dip G2-M: 17.58 % at 152.97
Dip S: 27.63 % G2/G1: 2.01
Dip %CY: 2.31
CELL CYCLE ANALYSIS

G0/G1: 61.45%
S-phase: 29.63%
G2-M: 8.92%
CELL CYCLE ANALYSIS:

just after release

2 hours

4 hours

6 hours

8 hours

RELEASE FROM INHIBITION OF DNA SYNTHESIS
HEADS UP ON DNA ANALYSIS

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CELL CYCLE ANALYSIS:

* WOOPS! THESE ARE ALL THE SAME DATA FILE
HEADS UP ON DNA ANALYSIS

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- AGGREGATES
  - REAL CLUMPS
  - PSEUDO CLUMPS = COINCIDENT EVENTS
What can you not tell from this histogram?
TIME OF FLIGHT (WIDE LASER BEAM)

\[ V = \left(\frac{4}{3}\right) \pi r^3 \]

modified after Ormerod, 1990

\[
\begin{align*}
\text{a} : & \quad [1] \quad 2 \quad 2 \\
\text{h} : & \quad [1] \quad 2 \quad 2 \\
\text{w} : & \quad \text{lb} + [1] \quad \text{lb} + 1.26^* \quad \text{lb} + 2
\end{align*}
\]
TIME OF FLIGHT (NARROW LASER BEAM)

modified after Ormerod, 1990

\[ V = \left( \frac{4}{3} \right) \pi r^3 \]

\[ a : \begin{array}{ccc} [1] & 2 & 2 \\ h : \begin{array}{ccc} [1] & 1.26^* & 1 \\ w : \begin{array}{ccc} lb+[1] & lb+1.26 & lb+2 \end{array} \end{array} \]
GATING TO "REMOVE" CLUMPS (10µm vs 20µm BEAM)

Coulter Cytometer: 10µm beam

BD Cytometer: 20 µm beam

note: these are different cell samples
GATING TO “REMOVE” CLUMPS (w. 20µm BEAM)

area vs width

area vs height

ungated

gated on area vs width
MATHEMATICAL MODELING TO "REMOVE" CLUMPS
FULL ANALYSIS

DIPLOID: 100.00 %
Dip G0-G1: 61.09 % at 76.28
Dip G2-M: 10.09 % at 152.84
Dip S: 28.81 % G2/G1: 2.00
Dip %CY: 2.30

Extra Pop: %
Debris: 3.15 %
Aggregates: 19.32 %
Modeled Events: 14578
RCS: 4.175
Diploid B.A.D.: 8.48 %
VIABILITY ASSAYS: NOT AS EASY AS YOU MIGHT THINK

XENOPUS SPLEEN LYMPHOCYTES

PROPIDIUM IODIDE FLUORESCENCE

"LIVE" GATE

2.7% DEAD

"DEAD" GATE

91% DEAD
SPERM SORTING ACCORDING TO DNA CONTENT

July, 1999

This slide used to show cows: Now it is us.

a flow cytometer

Things to think about:
• other things besides cells can be analyzed for DNA content e.g. sperm or chromosomes or molecules
• even if you try to hide behind a flow cytometer, you still cannot avoid ethical issues
• there is more to life than flow cytometry