institutCurie
Ensemble, prenons le cancer de vitesse.

Multiparametric analysis of solid tumors using flow and Image cytometry

Zofia Maciorowski
Institut Curie, Paris, France

BD

Nordic BD FACS™ Users Meeting
February 3-4 2009
Aronsborg, Stockholm, Sweden

Overview

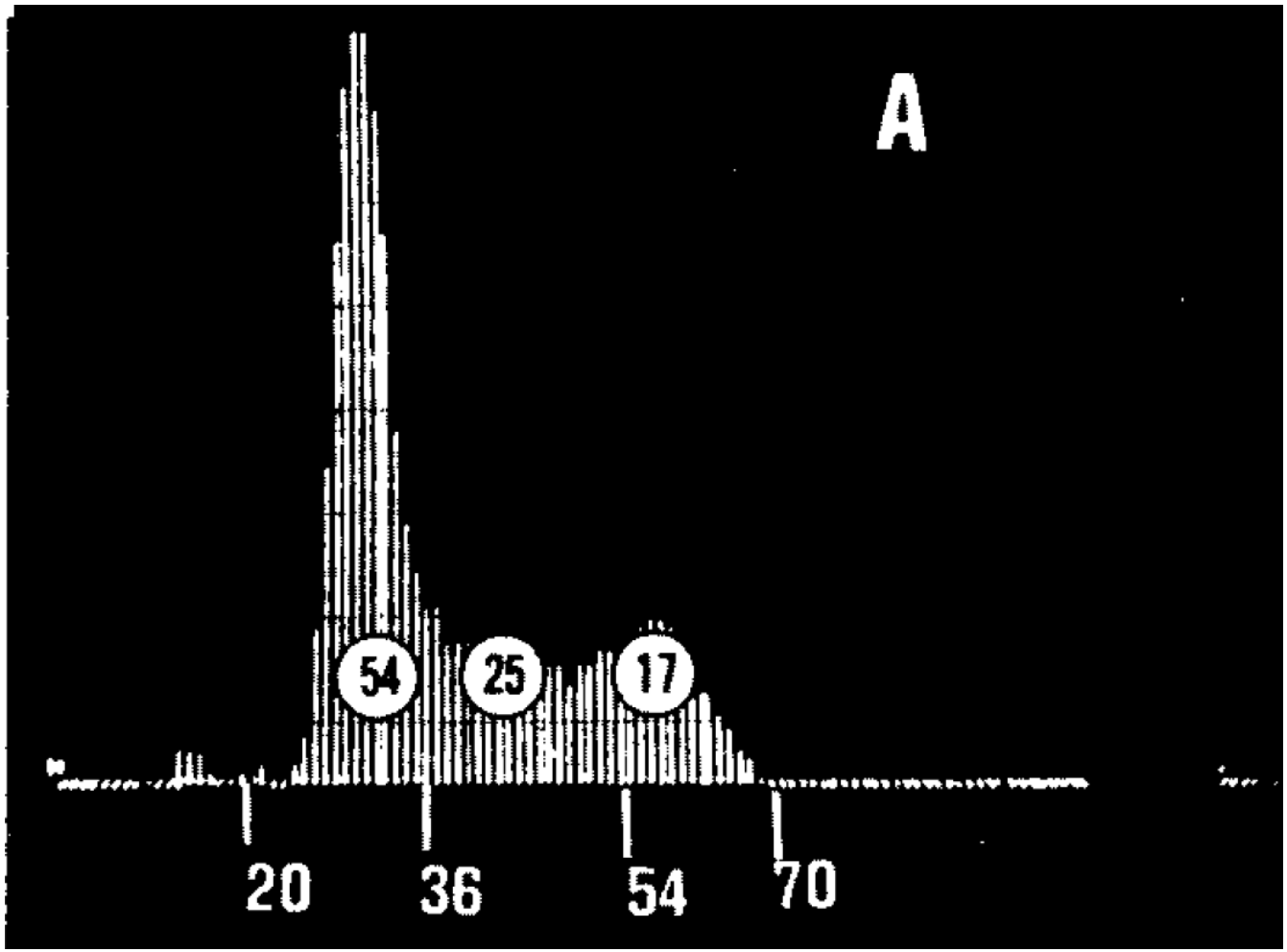
- **History and basic theory of DNA and Sphase in solid tumors**
- **Technical problems encountered in the preparation of solid tumors**
- **Multiparameter analysis of solid tumors using flow cytometry**
- **Multiparameter analysis of solid tumors using image cytometry**

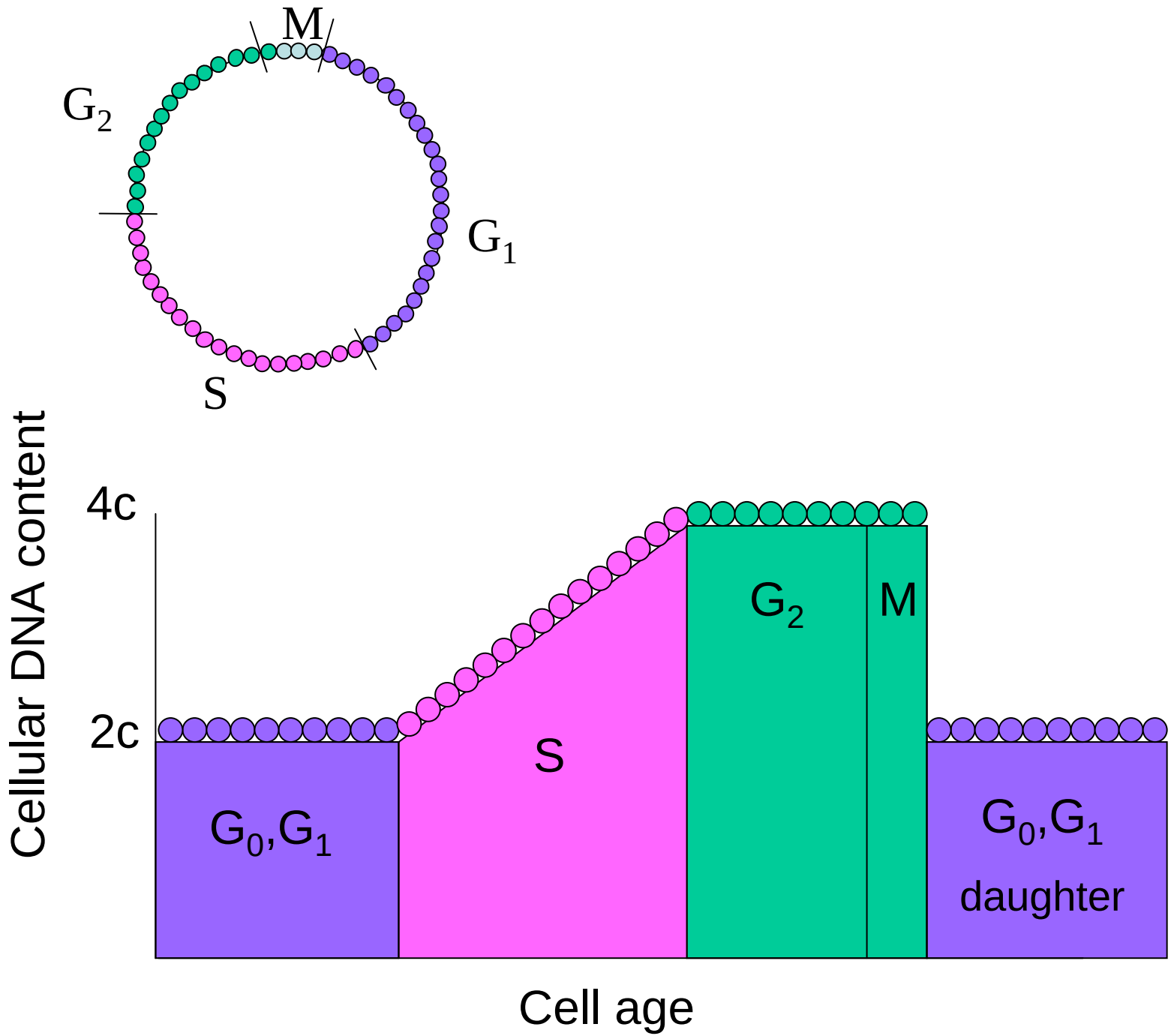
1975

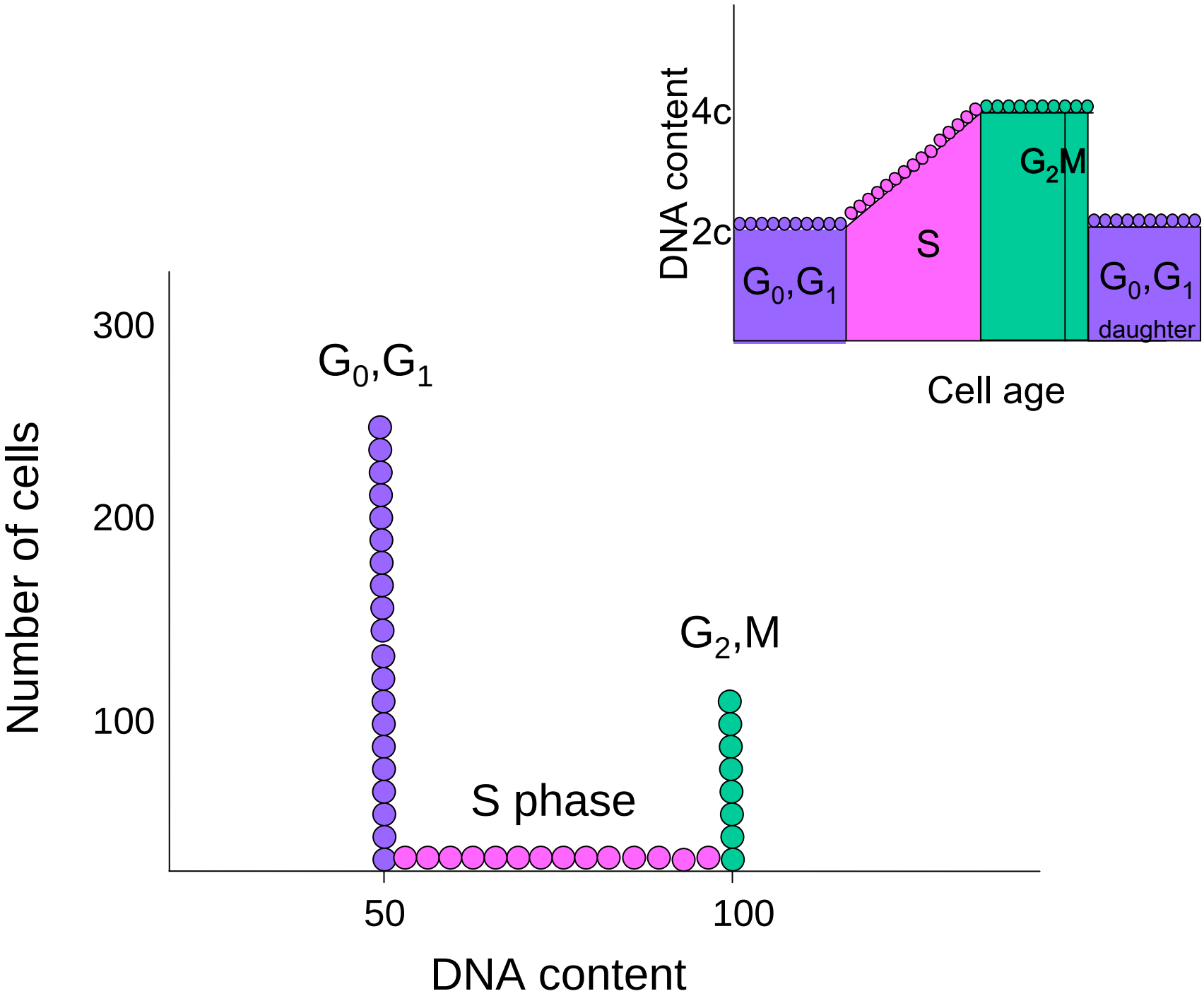
RAPID FLOW CYTOFLUOROMETRIC ANALYSIS OF MAMMALIAN CELL CYCLE BY PROPIDIUM IODIDE STAINING

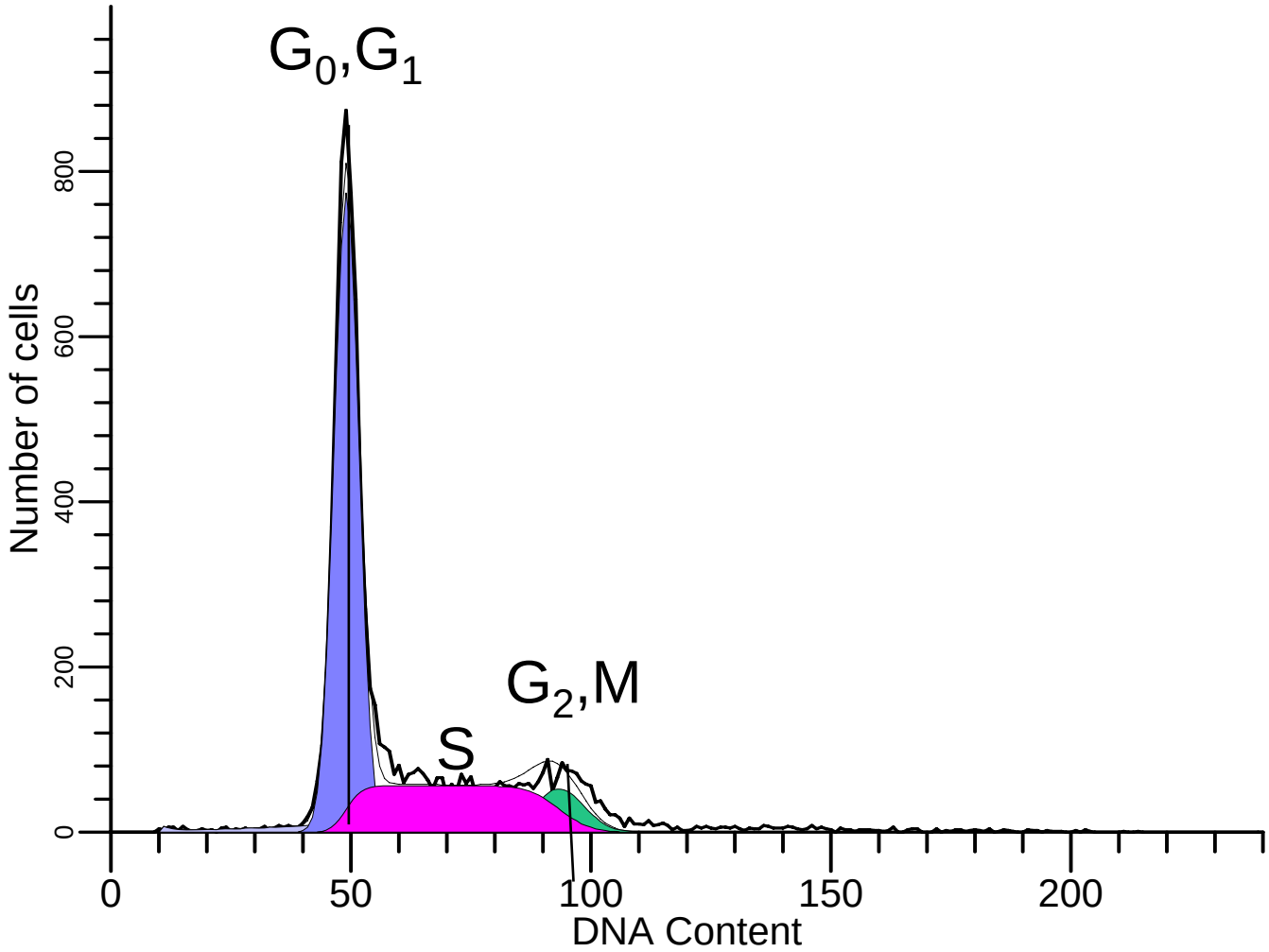
AWTAR KRISHAN. From the Sidney Farber Cancer Center and Harvard Medical School, Boston, Massachusetts 02115

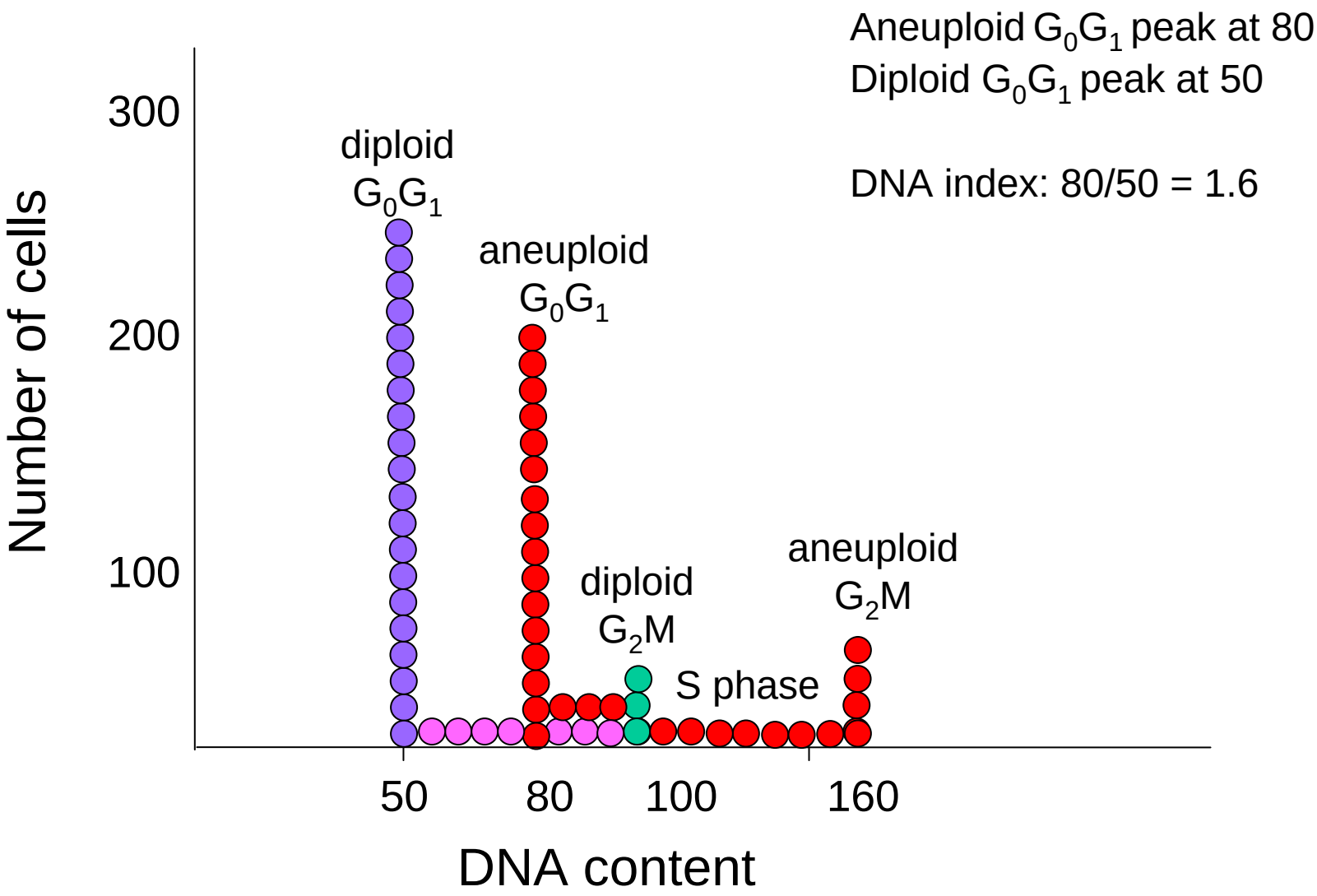
THE JOURNAL OF CELL BIOLOGY · VOLUME 66, 1975 · pages 188-193

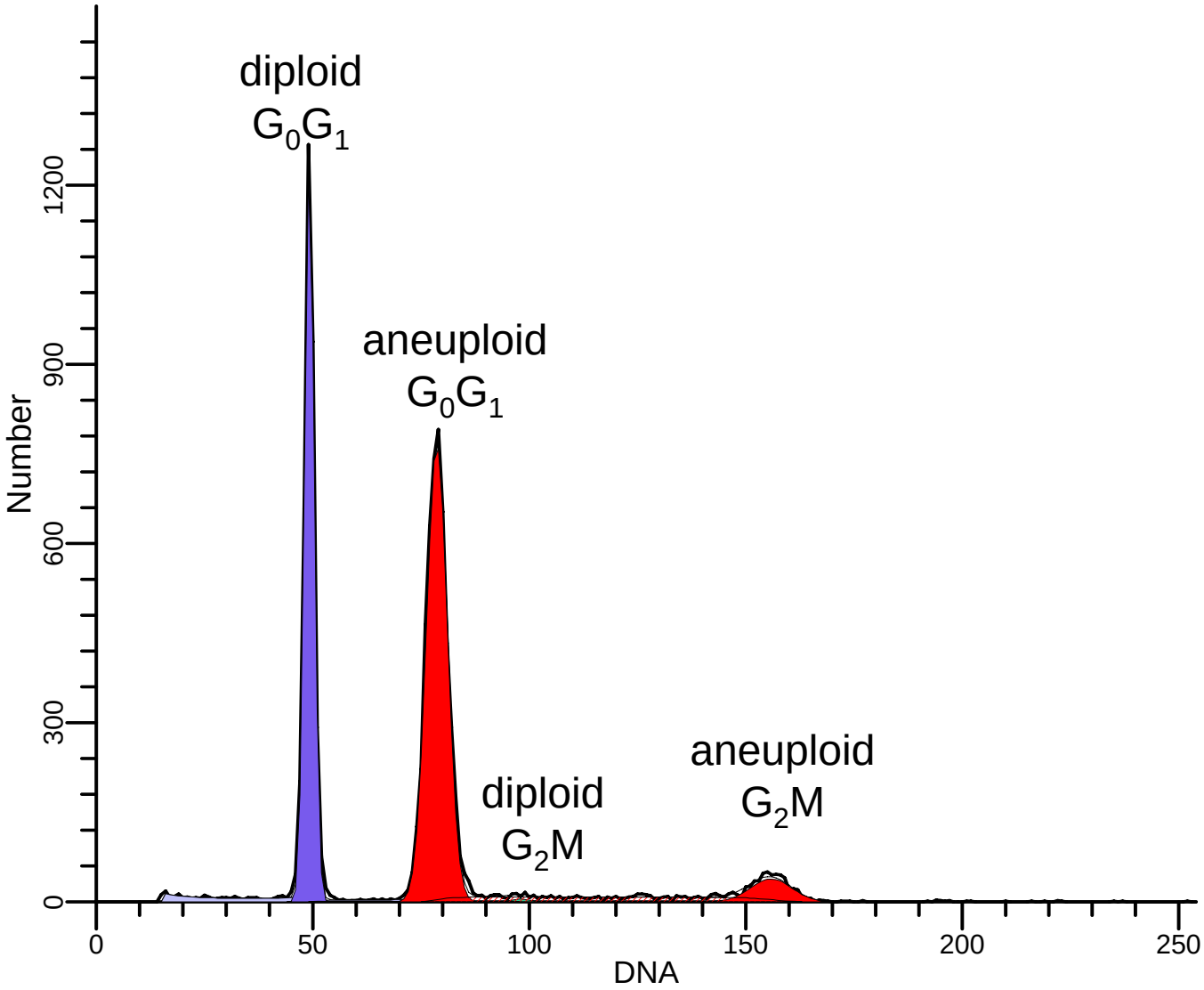



M
G2
G1
S
Cellular DNA content
4c
2c
G0,G1
S
G2
M
G0,G1
daughter
Cell age

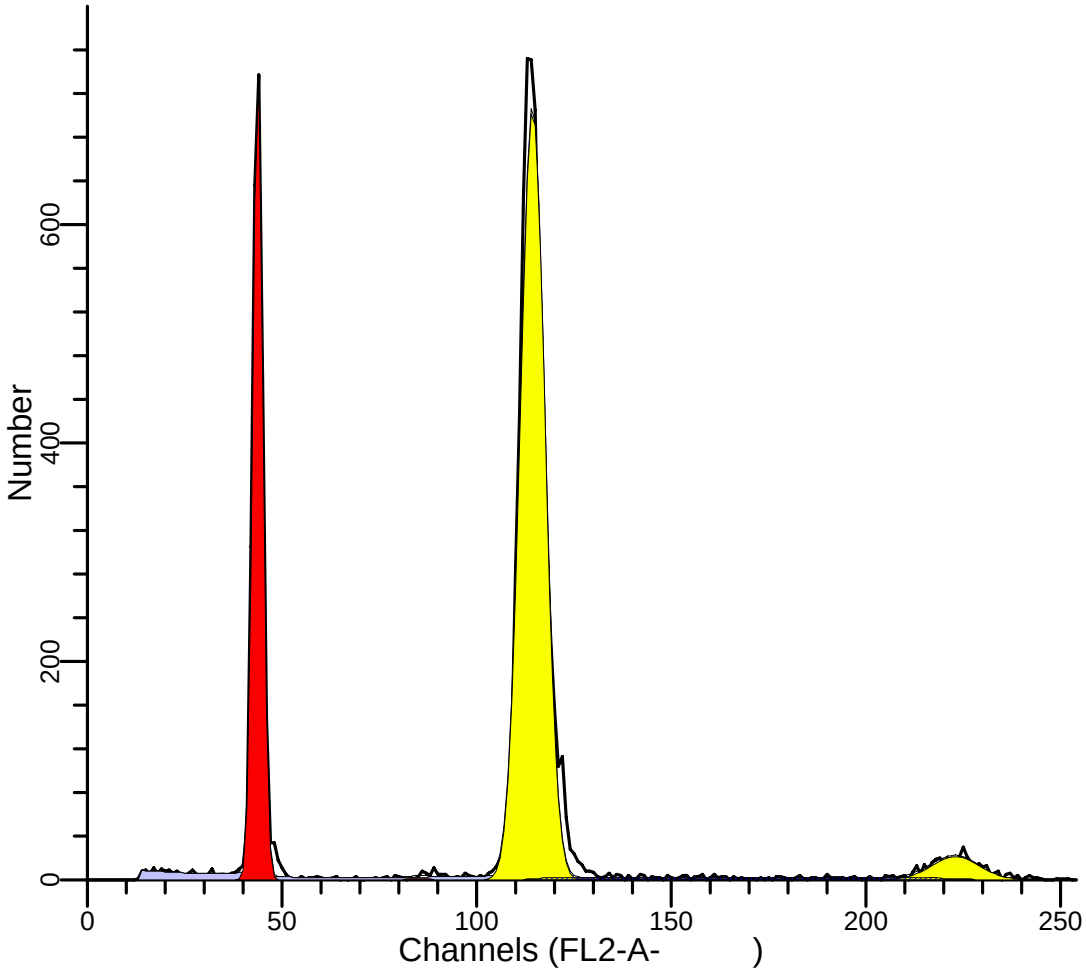

DNA content
4c
2c
G_0,G_1
S
G_2M
G_0,G_1
daughter
Cell age
Number of cells
300
200
100
G_0,G_1
S phase
G_2,M
50
100
DNA content


G0,G1
S
G2,M
Number of cells
0
200
400
600
800
0
50
100
150
200
DNA Content


Aneuploid G_0G_1 peak at 80
Diploid G_0G_1 peak at 50
DNA index: 80/50 = 1.6
Number of cells
300
200
100
diploid
G_0G_1
aneuploid
G_0G_1
diploid
G_2M
S phase
aneuploid
G_2M
50
80
100
160
DNA content


diploid
G_0G_1
aneuploid
G_0G_1
diploid
G_2M
aneuploid
G_2M
Number
0
300
600
900
1200
0
50
100
150
200
250
DNA

Aneuploid tumor



File analyzed: 9308152
Date analyzed: 16-Nov-2001
Model: 2Dn0n_DSD_ASD
Analysis type: Manual analysis

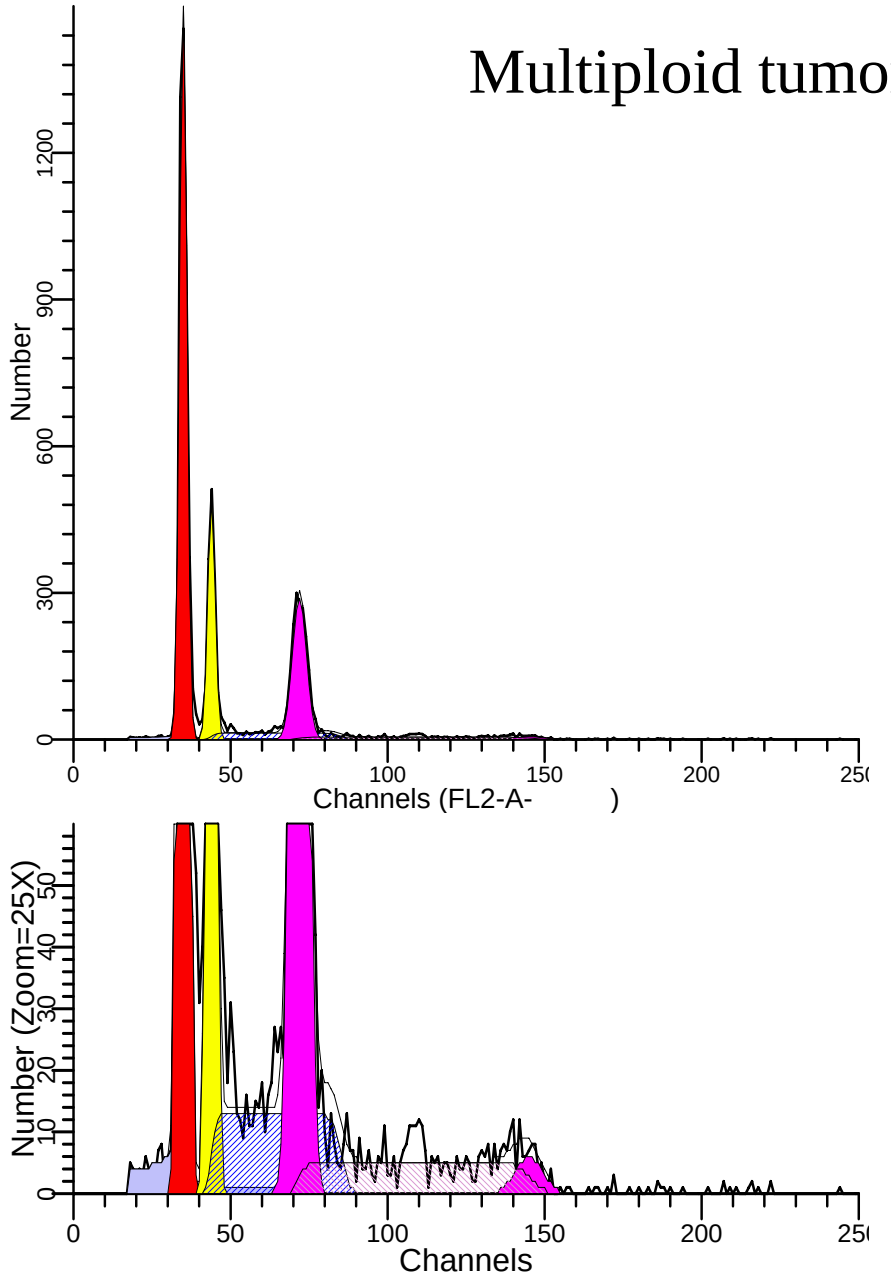
Diploid: 27.97 %
Dip G1: 99.64 % at 43.76
Dip G2: 0.36 % at 85.34
Dip S: 0.00 % G2/G1: 1.95
%CV: 2.80

Aneuploid 1: 72.03 %
An1 G1: 90.95 % at 114.36
An1 G2: 5.35 % at 222.99
An1 S: **3.70** % G2/G1: 1.95
%CV: 2.73 DI: **2.61**

Total Aneuploid S-Phase: 3.70 %
Total S-Phase: 2.67 %
Total B.A.D.: 2.64 % no aggs

Debris: 4.95 %
Aggregates: 0.00 %
Modeled events: 8892
All cycle events: 8451
Cycle events per channel: 47
RCS: 3.220

Multiploid tumor



File analyzed: 9308144
Date analyzed: 16-Nov-2001
Model: 3Dn0n_Dnn_ASD_TSF
Analysis type: Manual analysis

Diploid: 56.76 %
Dip G1: 92.00 % at 34.95
Dip G2: 8.00 % at 71.92
Dip S: 0.00 % G2/G1: 2.06
%CV: 3.16

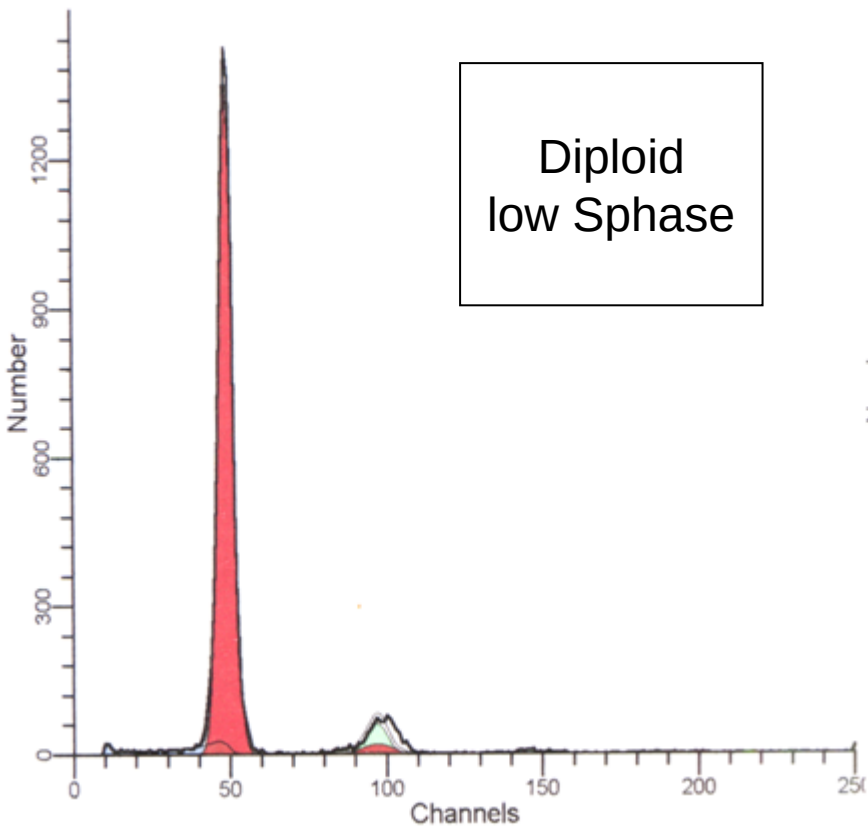
Aneuploid 1: 23.37 %
An1 G1: 73.51 % at 43.92
An1 G2: 0.00 % at 85.20
An1 S: 26.49 % G2/G1: 1.94
%CV: 2.61 **DI: 1.26**

Tetraploid: 19.87 %
An2 G1: 73.16 % at 71.92
An2 G2: 3.90 % at 145.14
An2 S: 22.94 % G2/G1: 2.02
%CV: 3.07 **DI: 2.06**

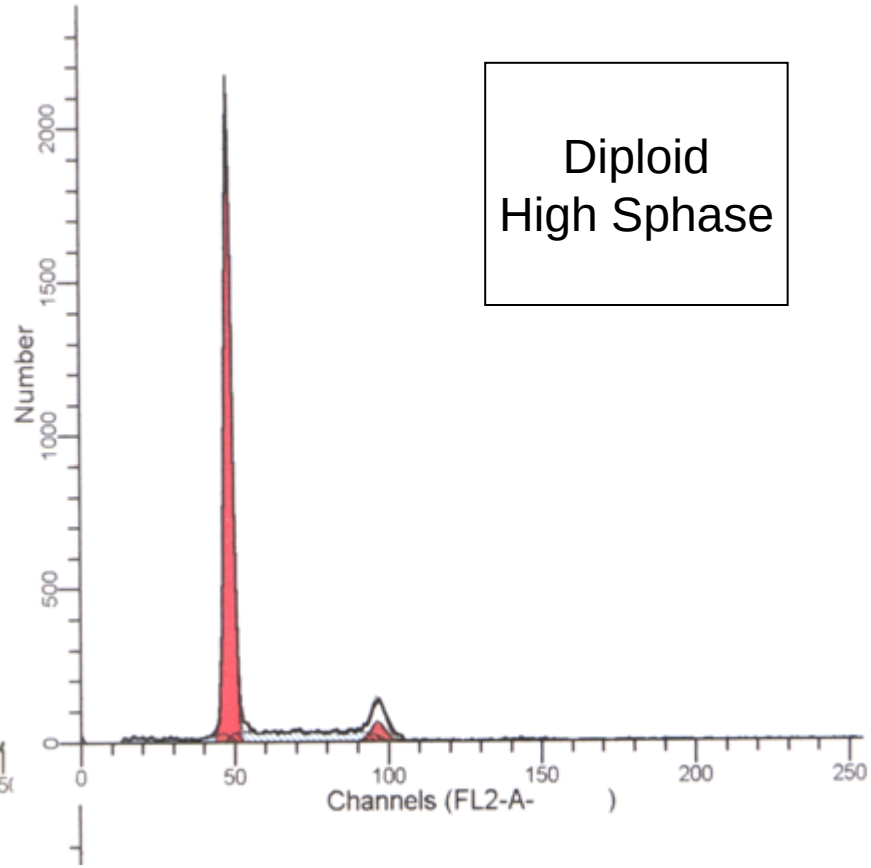
Total Aneuploid S-Phase: 24.86 %
Total S-Phase: 10.75 %
Total B.A.D.: 0.86 % no aggs

Debris: 1.90 %
Aggregates: 0.00 %
Modeled events: 8598
All cycle events: 8435
Cycle events per channel: 76
RCS: 2.934

Breast Cancer

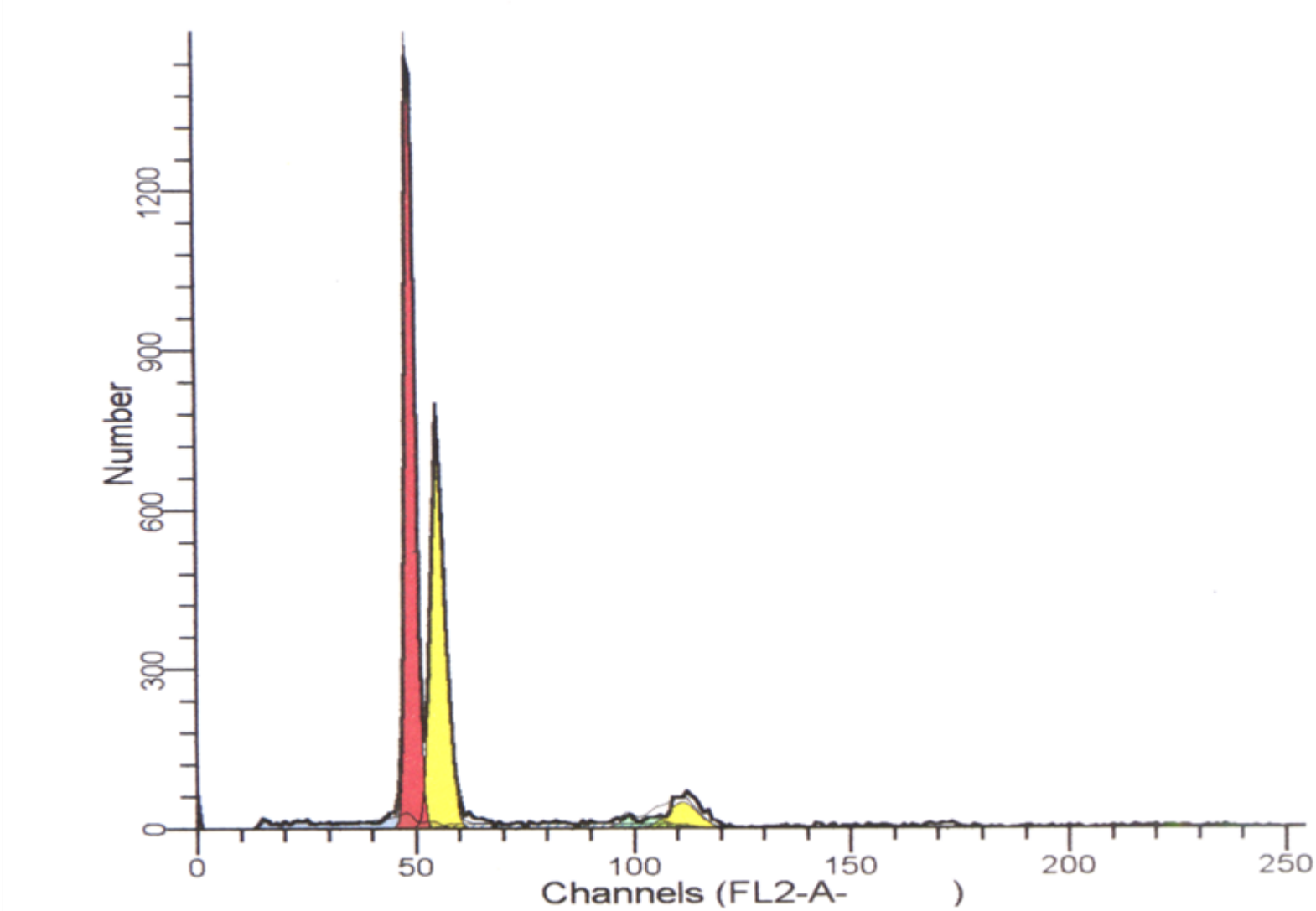


Diploid
low Sphase



Diploid
High Sphase

DNA index 1.1-1.3: diploid or aneuploid?



DNA Cytometry Consensus Conference

Guidelines for Implementation of Clinical DNA Cytometry

Shankey et al, Cytometry 14:472-477 (1993)

Consensus Review of the Clinical Utility of DNA Cytometry in Carcinoma of the Breast

Hedley et al, Cytometry 14:482-485 (1993)

DNA Cytometry Consensus Conference

Problems:

- **lack of agreement between clinical studies**
- **Due to technical factors**
- **Preparation and analysis procedures not standardized**

Recommendations:

- **Sampling: representative of tumor cells (20% minimum)**
- **Stoichiometric binding of dyes**
- **Diploid standards for ploidy determination**
- **Individual laboratory determination of cutoffs for sphase**
- **Development of multiparameter analysis**

Optimizing Flow Cytometric DNA Ploidy and S-Phase Fraction as Independent Prognostic Markers for Node-Negative Breast Cancer Specimens

C.B. Bagwell, G.M. Clark, F. Spyratos, A. Chassevent, P.-O. Bendahl, O. Stål, D. Killander, M.L. Jourdan, S. Romain, B. Hunsberger, and B. Baldetorp

Cytometry (Communications in Clinical Cytometry) 46:121–135 (2001)

- **Lead to standardization and rules for sphase analysis of DNA histograms.**
- **Training and rules are available on the Verity software site.**

ModFit LT Rule-Based Training System

Verity Software

Part A:	**What is a DNA Histogram? – Prerequisite tutorial**
Part B:	**Introduction / Training overview**
Part C:	**ModFit LT overview**
Part D:	**AutoAnalysis: how it works**
Part E:	**Peak Finder, Auto Analysis and Configuration settings.**
Part F:	**General Strategy for File Analysis and Review**
Part G:	**DNA Diploid Files - Analysis and Review**
Part H:	**DNA Tetraploid Files - Analysis and Review**
Part I:	**DNA Aneuploid Files - Analysis and Review**
Part J:	**DNA Multiploid / Hypodiploid Files - Analysis and Review**
Part K:	**Phase 5 - Databasing and Printing Reports**
Part L:	**Proficiency Exam**
Part M:	**Advanced Analysis Techniques**

Appendix 1: Rules for Obtaining High Quality DNA Histograms and Optimizing Correlation of S-phase Estimates Between Operators.

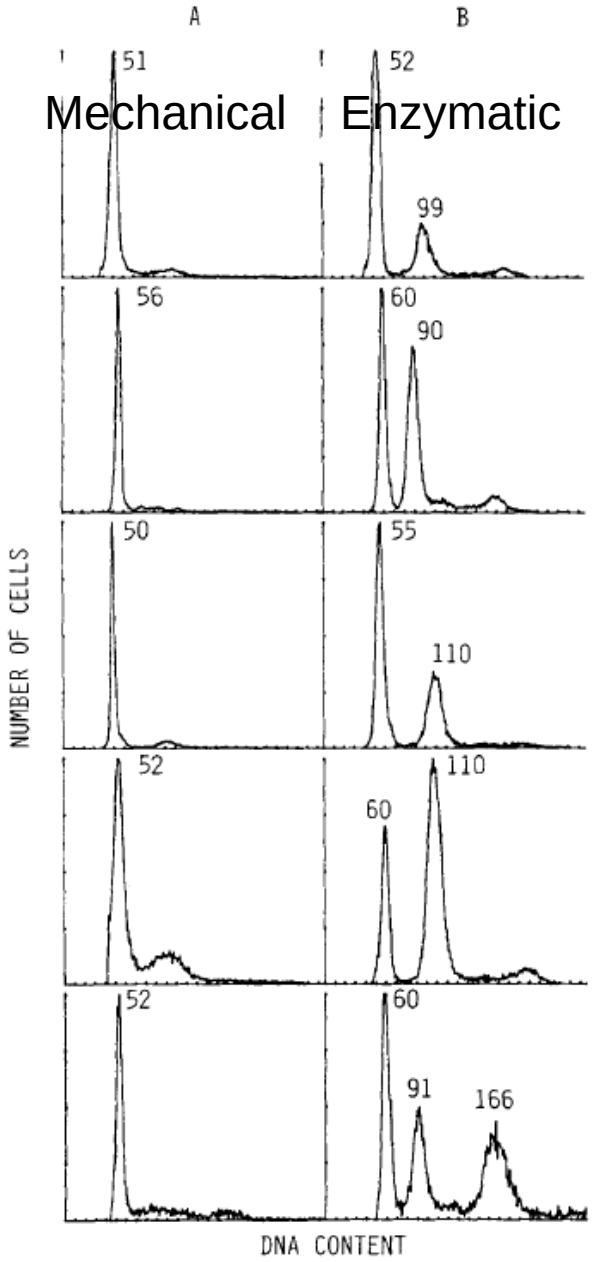
Solid tumor preparation and staining problems

- **flow cytometry requires single cell suspensions**
- **loss of populations of interest during tumor disaggregation**
- **staining differences between types of diploid cells**

loss of populations of interest during tumor disaggregation

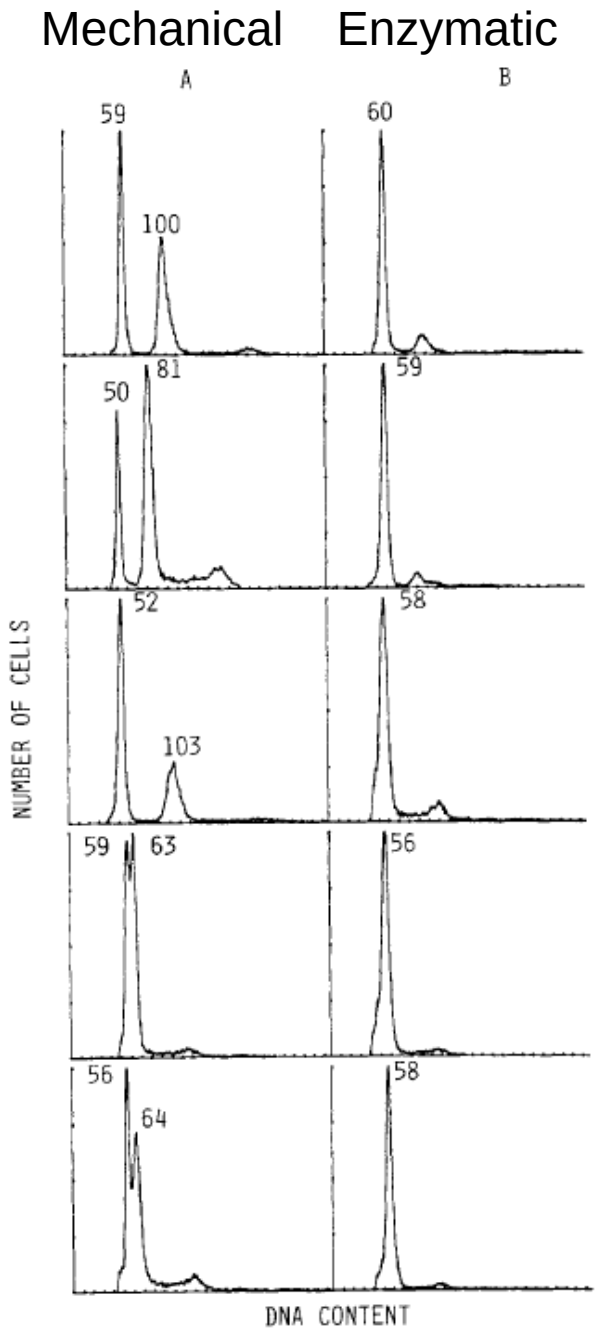
Squamous cell carcinomas of the head and neck

Disaggregation technique: Mechanical vs enzymatic



Colon Carcinoma

Disaggregation technique:
Mechanical vs enzymatic



Fine needle Aspirates (FNA)

Fine needle aspirates are dispersed mechanically by the needle.

Average yield in our experience is 3.1 x 10^6, median 1.4 x 10^6, with a range of 0.01 to 40 x 10^6. Trypan blue viability ranged from 5 to 80% viable. Naked nuclei were not unusual.

In FNA and pleural effusions , tumor cells of interest are often in small clumps. Clumps are not analyzed by flow cytometry

staining differences between types of diploid cells

Accessibility of DNA In Situ to Various Fluorochromes:
Relationship to Chromatin Changes During Erythroid Differentiation of Friend Leukemia Cells
Darzynkiewicz et al
Cytometry 5:355-363 (1984)

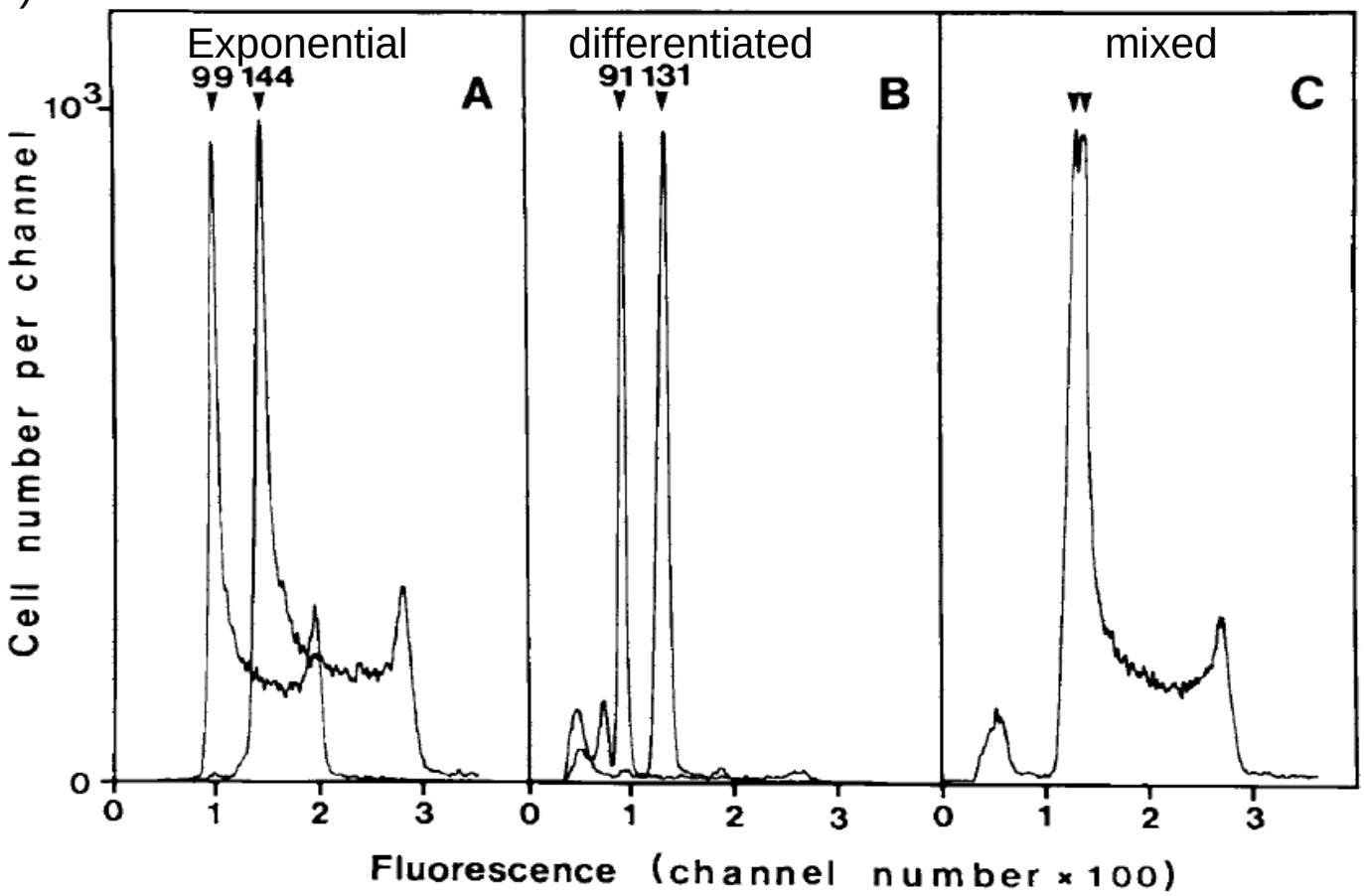


FIG. 1. Frequency distribution histograms representing fluorescence of FL cells stained with 4'6-diamidino-2-phenylindole (DAPI). A) Exponentially growing cells before (peak value = 99) and after treatment with 0.1N HCl (peak value = 144). B) Differentiated cells before (peak = 91) and after extraction with 0.1N HCl (peak = 131). C) Differentiated cells were mixed with exponentially growing ones in 1:2 proportion, treated with 0.1N HCl, and stained. In these mixed cell populations, cells in G_1 exhibit different stainability, as manifested by the divided G_1 peak (arrow). One-step staining (without HCl-treatment) of mixed populations resulted also in a bimodal cell distribution within the G_1 peak in repeated experiments (not shown).

Pseudoaneuploid subpopulations detected in normal upper aerodigestive tract mucosa consistent with physiological apoptosis in normally differentiating squamous mucosa

El-Rayes et al

Otolaryngology-Head and Neck Surgery 131 no5, 633-638, 2004

Normal squamous mucosa

Squamous cell carcinoma head and neck

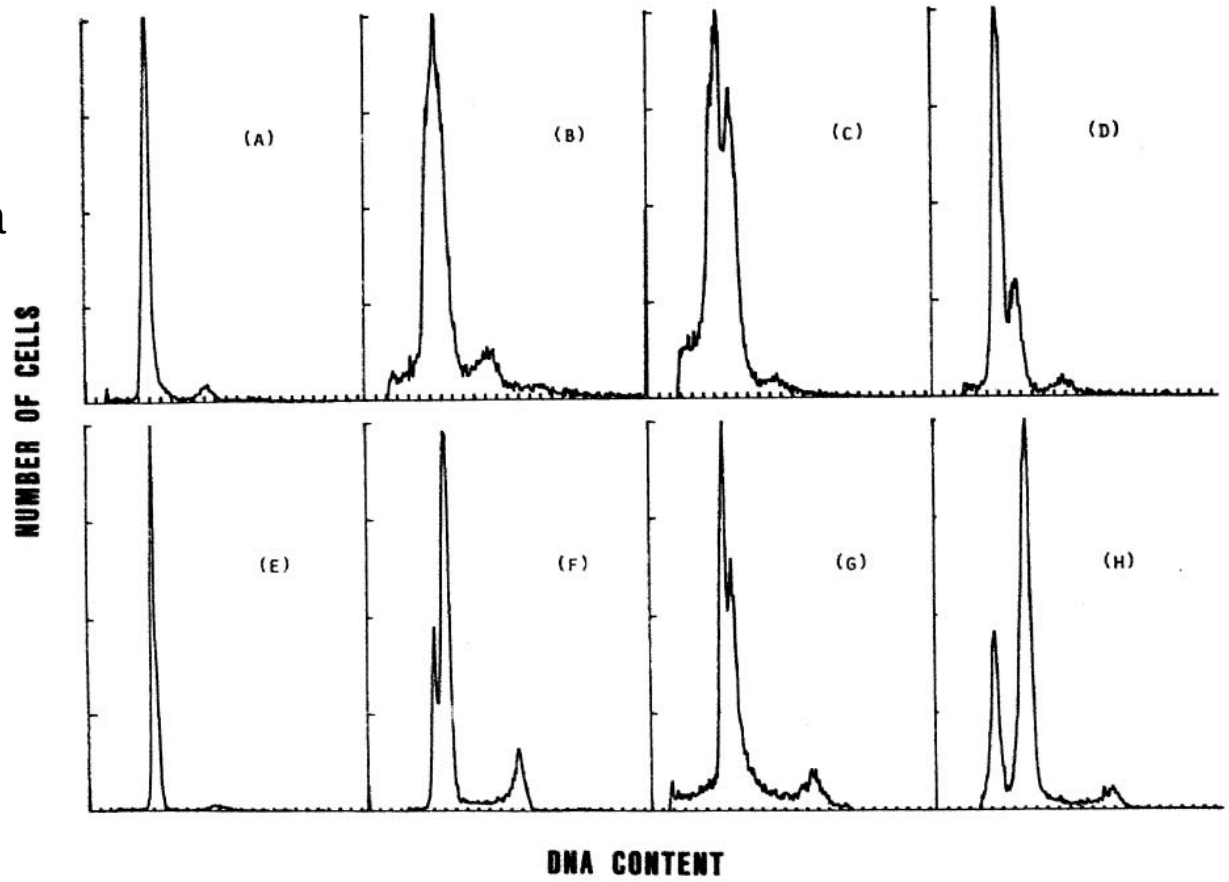


Fig 1. (**A-D**) DNA histograms from normal squamous mucosa. (**E-H**) DNA histograms from patients with SCCHN.

Advantages of multicolor analysis

- Distinguish between stromal, inflammatory and tumor cells
- Identify populations of tumor cells with poor prognosis
- Maximize information from small samples

Flow vs slide based image cytometry advantages

- **Flow cytometry**
  - Multicolor analysis of many cells
  - Can identify rare populations
  - Cells can be sorted for subsequent applications

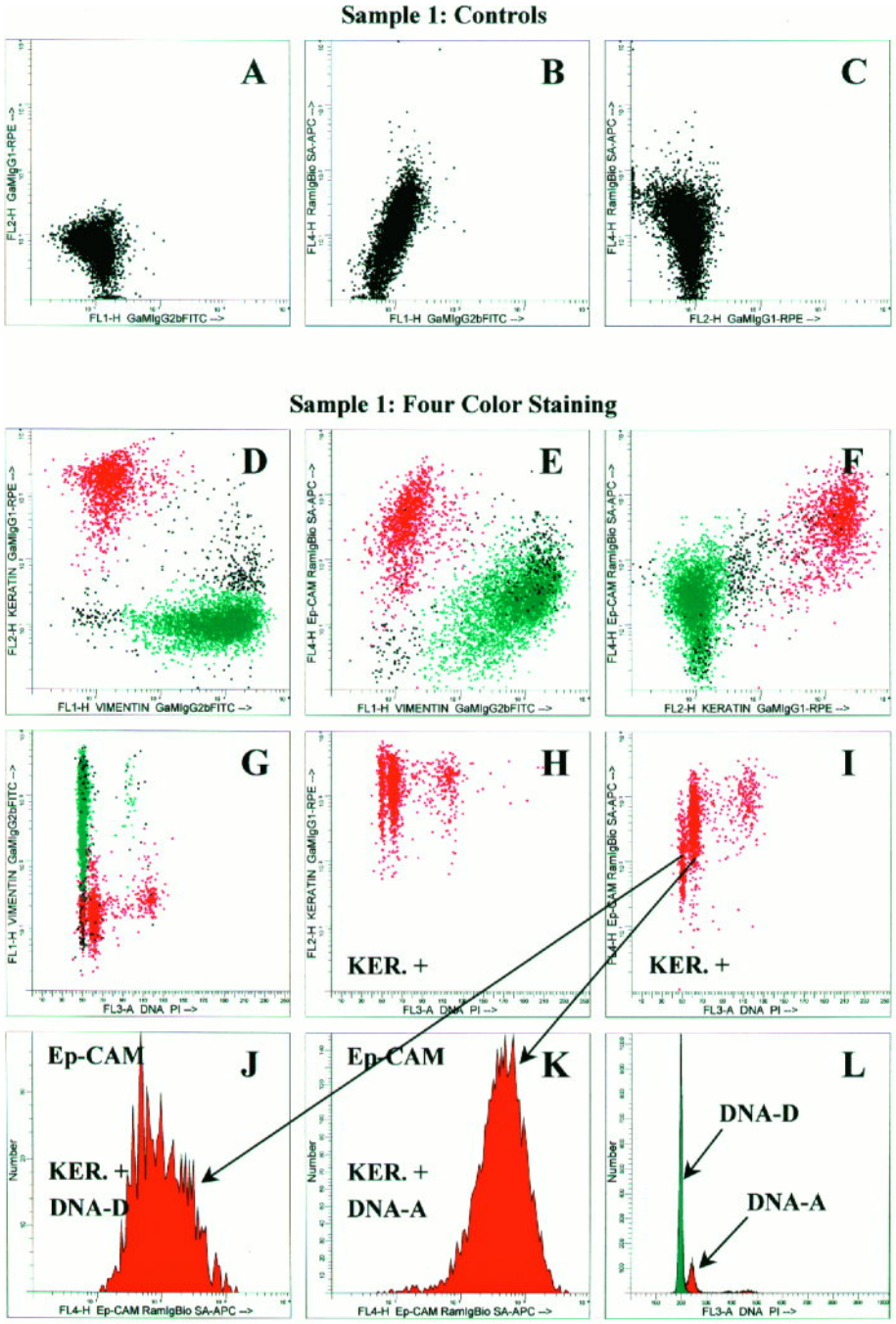
- **Slide based cytometry**
  - Multicolor analysis of fewer cells
  - Can see distribution and co-distribution of staining within cell
  - No loss of cells during processing
  - Can visualize questionable cells
  - Potential to restain slide for cell morphology for verification by pathologist

Flow vs slide based image cytometry disadvantages

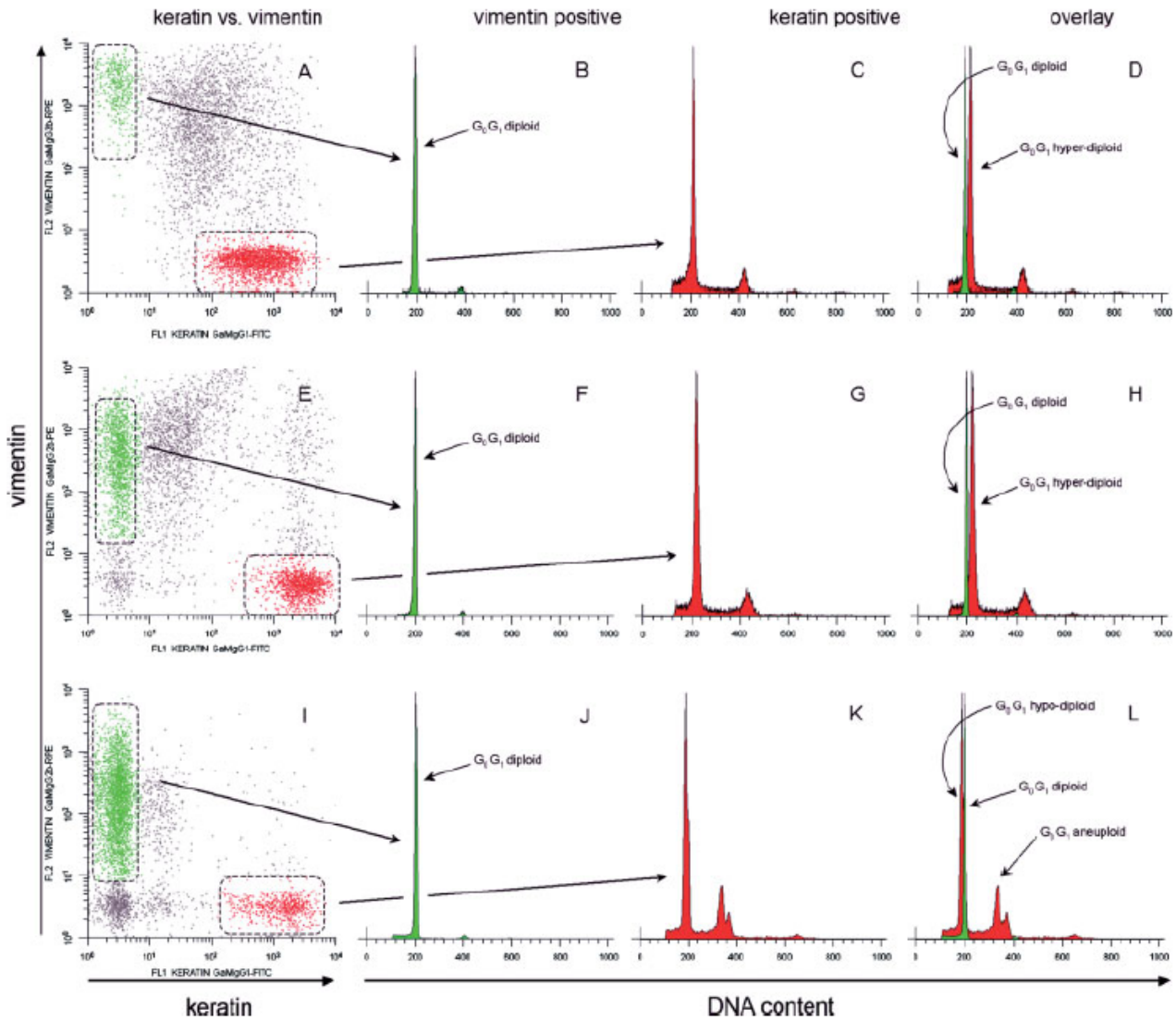
- **Flow cytometry**
  - Difficult to have good single cell preparations of solid tumors
  - Cells of interest often in clumps

- **Slide based cytometry**
  - Fewer cells analyzed
  - Fewer colors possible
  - Segmentation and quantitation of fluorescence difficult

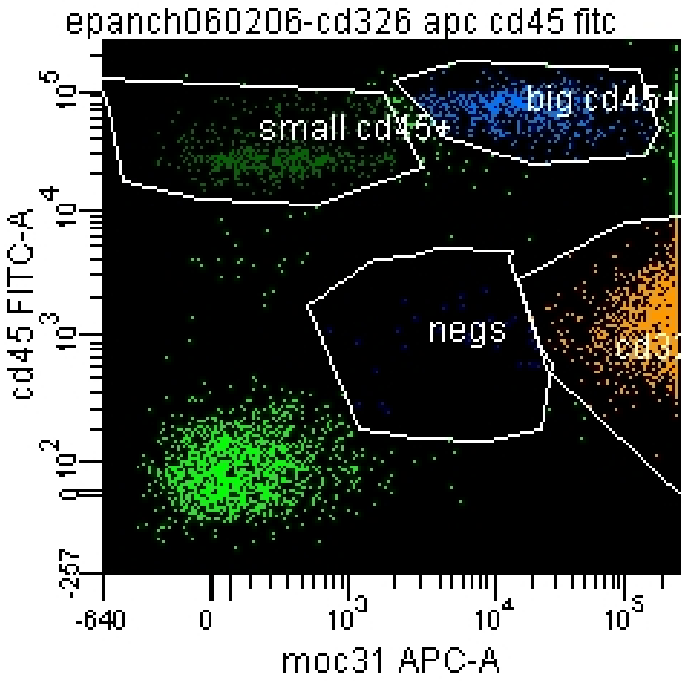
Four-color multiparameter DNA flow cytometric method to study phenotypic intratumor heterogeneity in cervical cancer
Corver et al Cytometry 39, 2, 1 Feb 2000, pp 96-107



High resolution multi-parameter DNA flow cytometry enables detection of tumor and stromal cell subpopulations in paraffin-embedded tissues
Corver et al
J. Pathology 2005:206,233-241



Cell sorting of pleural effusion stained with anti CD45 and anti-EpCAM

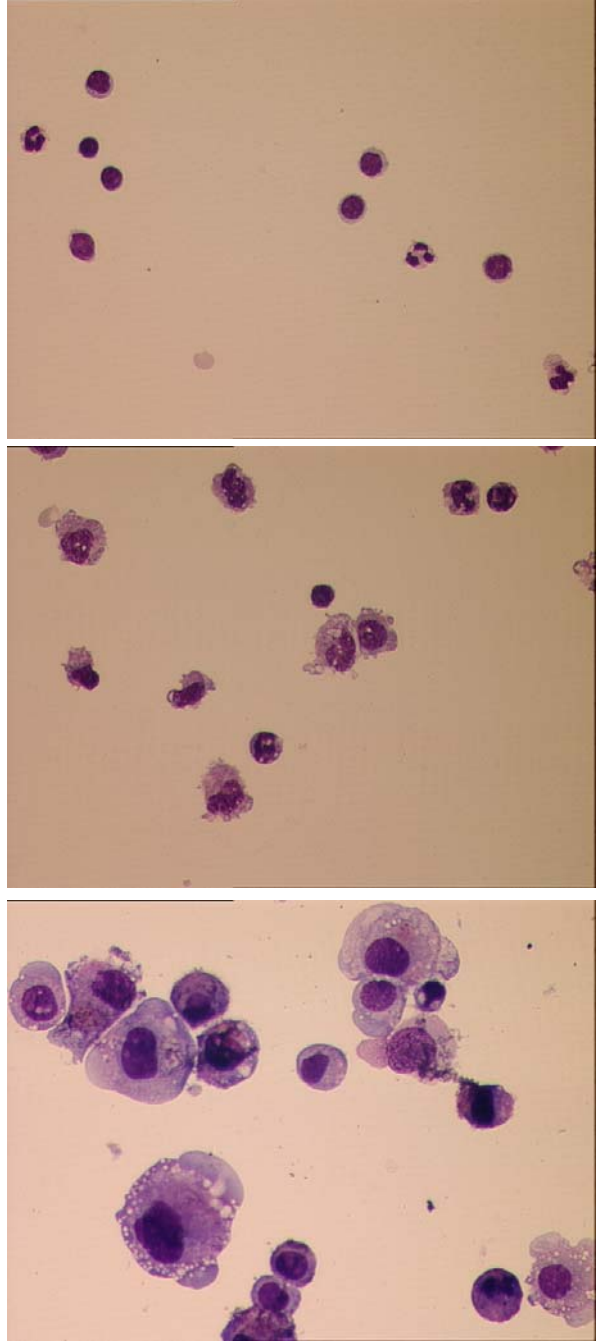


	#Events	%Parent
negs	29	0.5
cd326 +	2,797	48.8
small cd45+	572	10.0
big cd45+	589	10.3

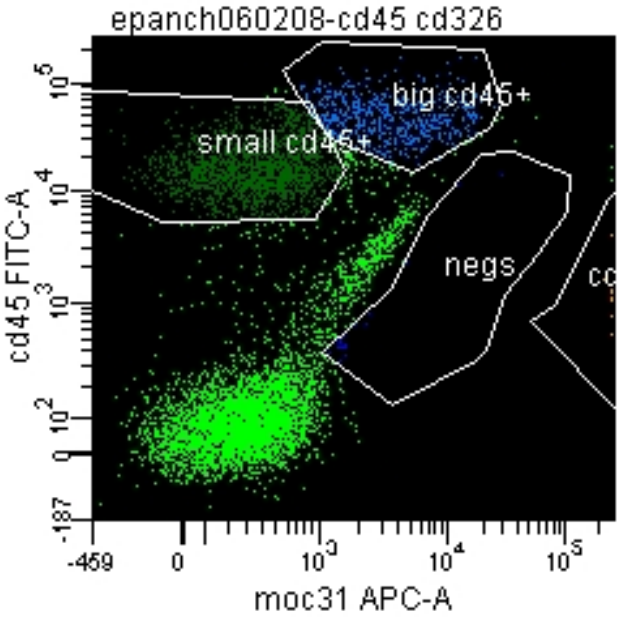
Small CD45+

large CD45+

EpCAM+



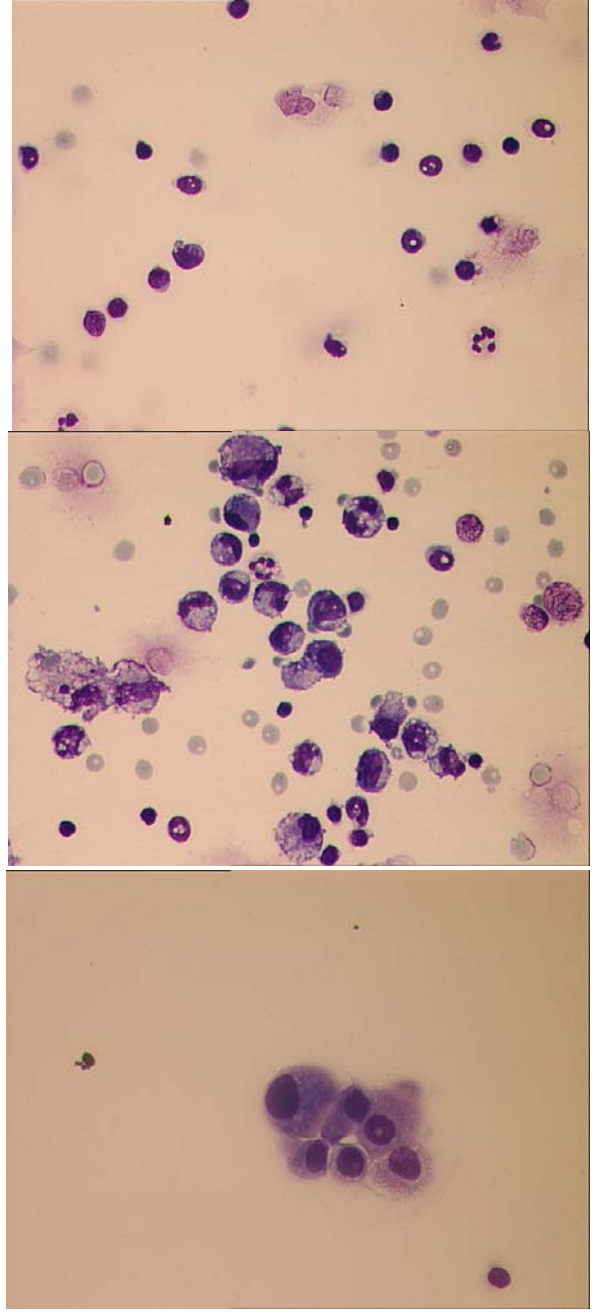
Cell sorting of pleural effusion stained with anti CD45 and anti-EpCAM



Small CD45+

large CD45+

EpCAM+



	#Events	%Parent
negs	90	0.3
cd326 +	32	0.1
small cd45+	7,334	23.4
big cd45+	2,852	9.1

Slide based DNA content

- Feulgen
- Fluorescent dyes: DAPI, PI

DNA Image Cytometry on Sections as Compared with Image Cytometry on Smears and Flow Cytometry in Melanoma
Klapperstuck and Wohlrab
Cytometry 25:82-89 (1996)

Feulgen stained melanoma tumors

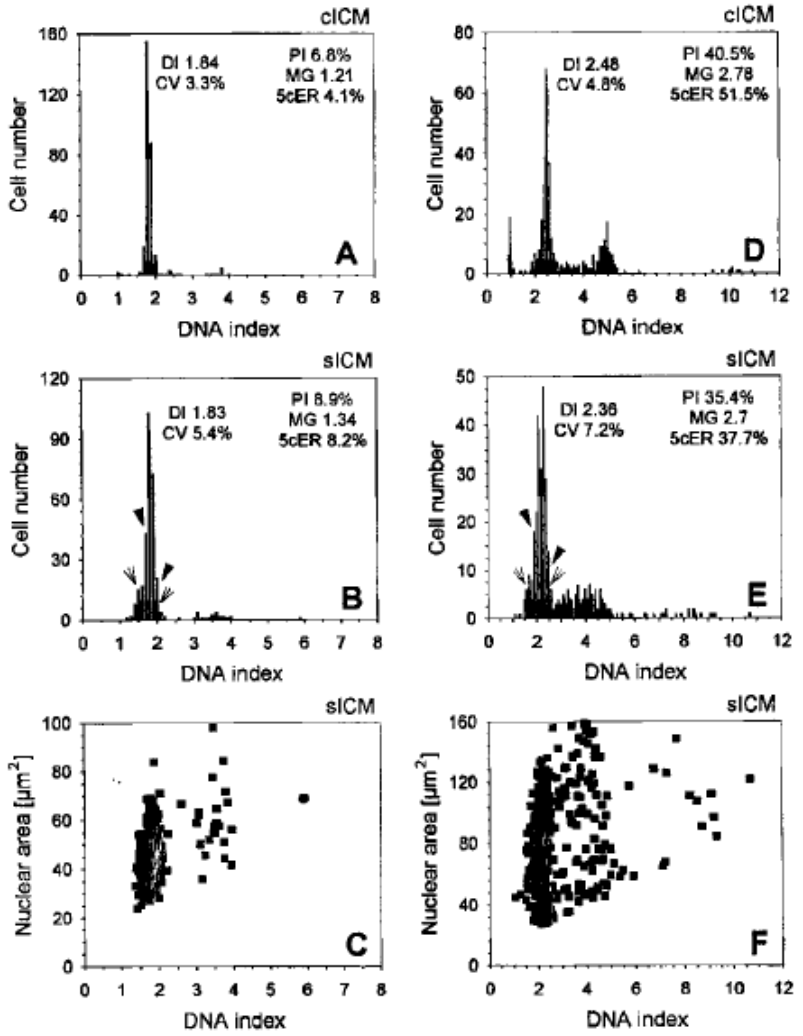


FIG. 1. Concordant DNA histograms obtained by cICM (**A,D**) and sICM (**B,E**) and DI-nuclear area dot plots obtained by sICM (**C,F**). Filled arrow heads mark the range used for DI and CV calculation (assumed to be intact nuclei). Open arrowheads mark the range used for the determination of the G0/G1 phase in percent. This includes a proportion of sectioned nuclei that is assumed to be part of G0/G1. Note that the DIs obtained from sections show no relationship to the nuclear area.

DAPI stained lymphocytes

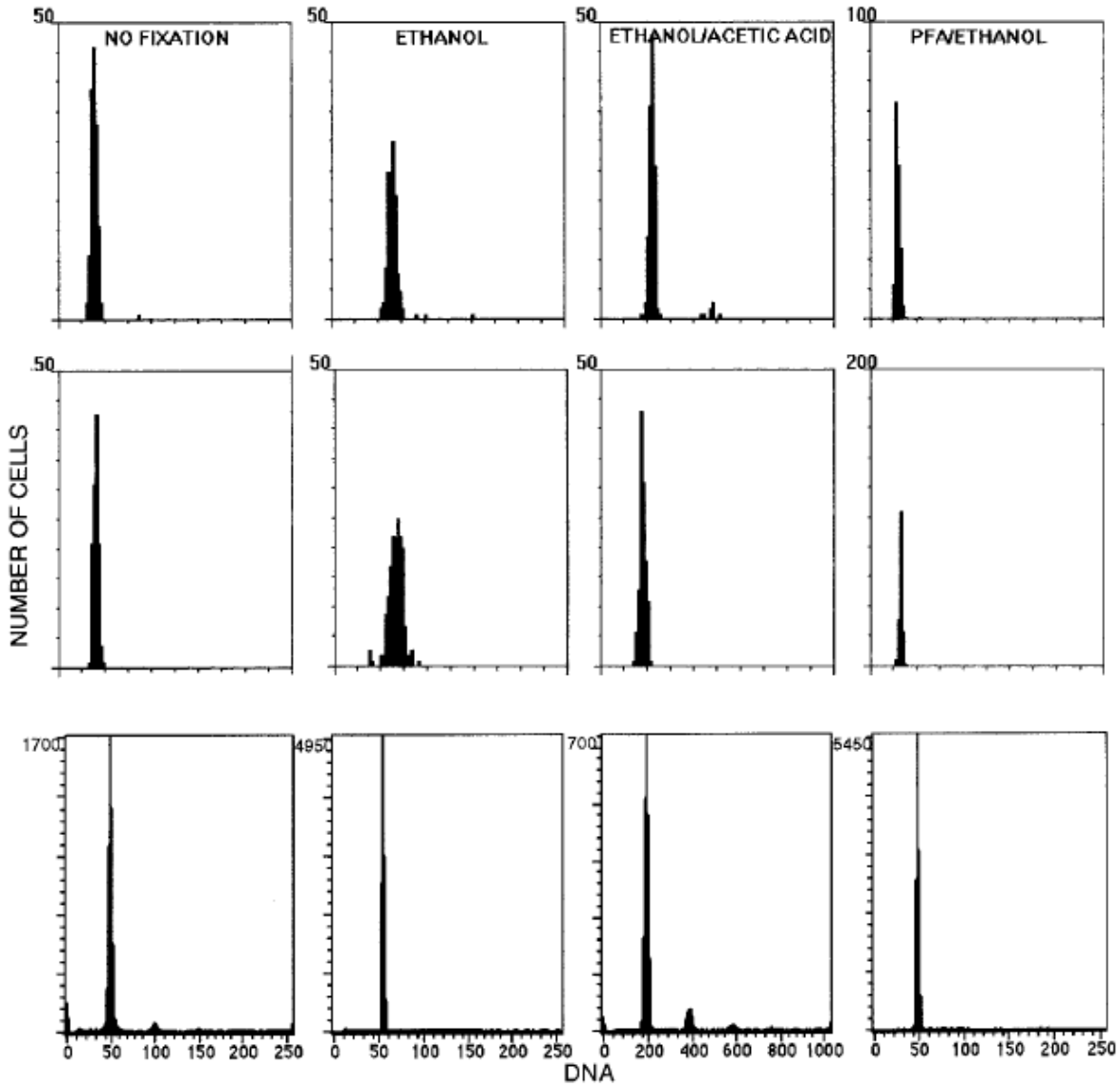


FIG. 1. Peripheral blood lymphocytes prepared as indicated: no fixation, ethanol, ethanol/acetic acid, or paraformaldehyde/ethanol fixation. The first two rows show image analysis histograms of either drop or cytospin slide preparations, and the last row shows the corresponding flow cytometry generated histograms.

DAPI stained breast carcinoma FNA

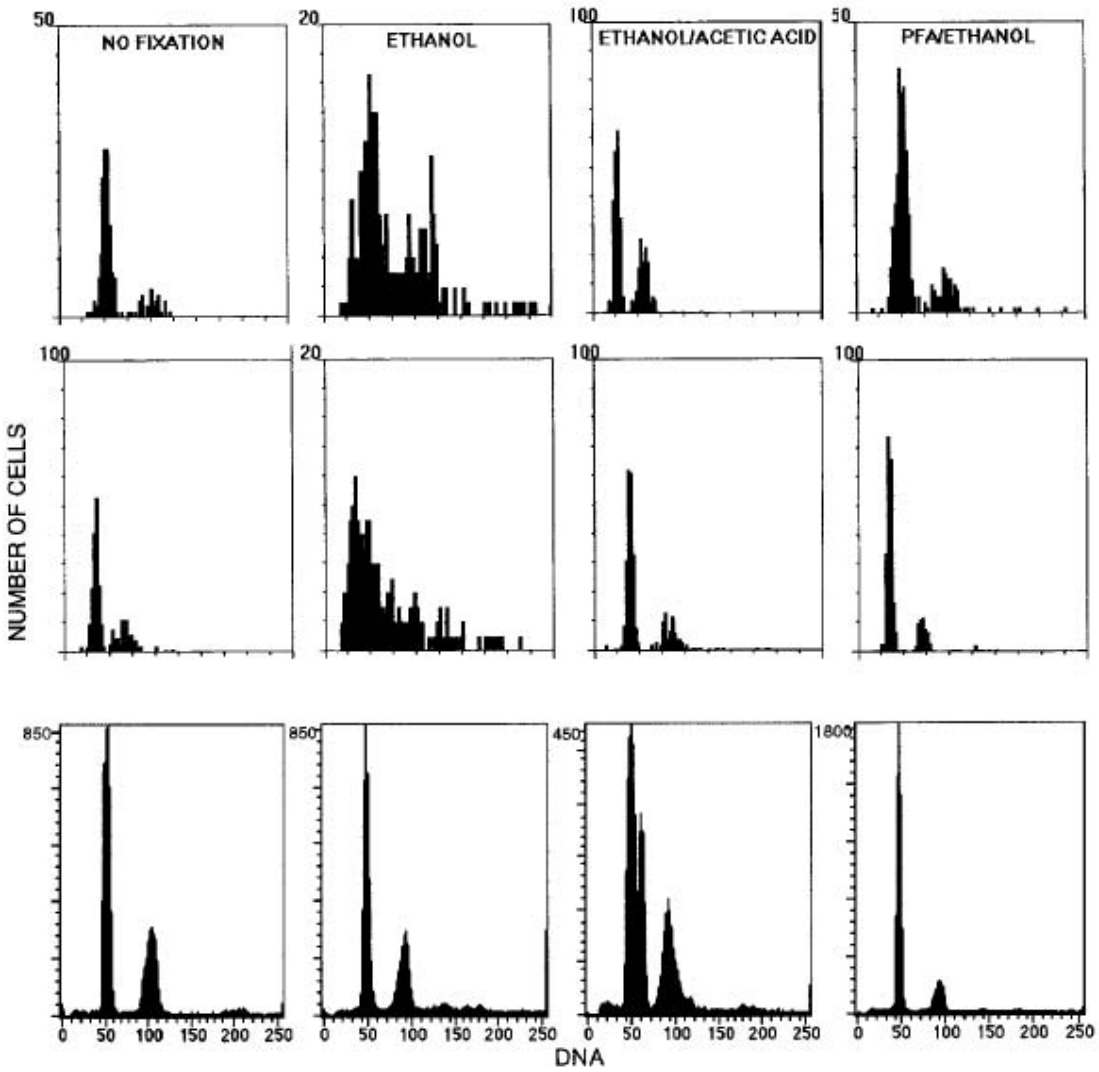


FIG. 2. **A,B:** Fine needle samples of two breast carcinomas prepared as indicated: no fixation, ethanol, ethanol/acetic acid, or paraformaldehyde/ethanol fixation. The first two rows show image analysis histograms of either drop or cytospin slide preparations, and the last row shows the corresponding flow cytometry generated histograms.

DAPI stained breast carcinoma FNA

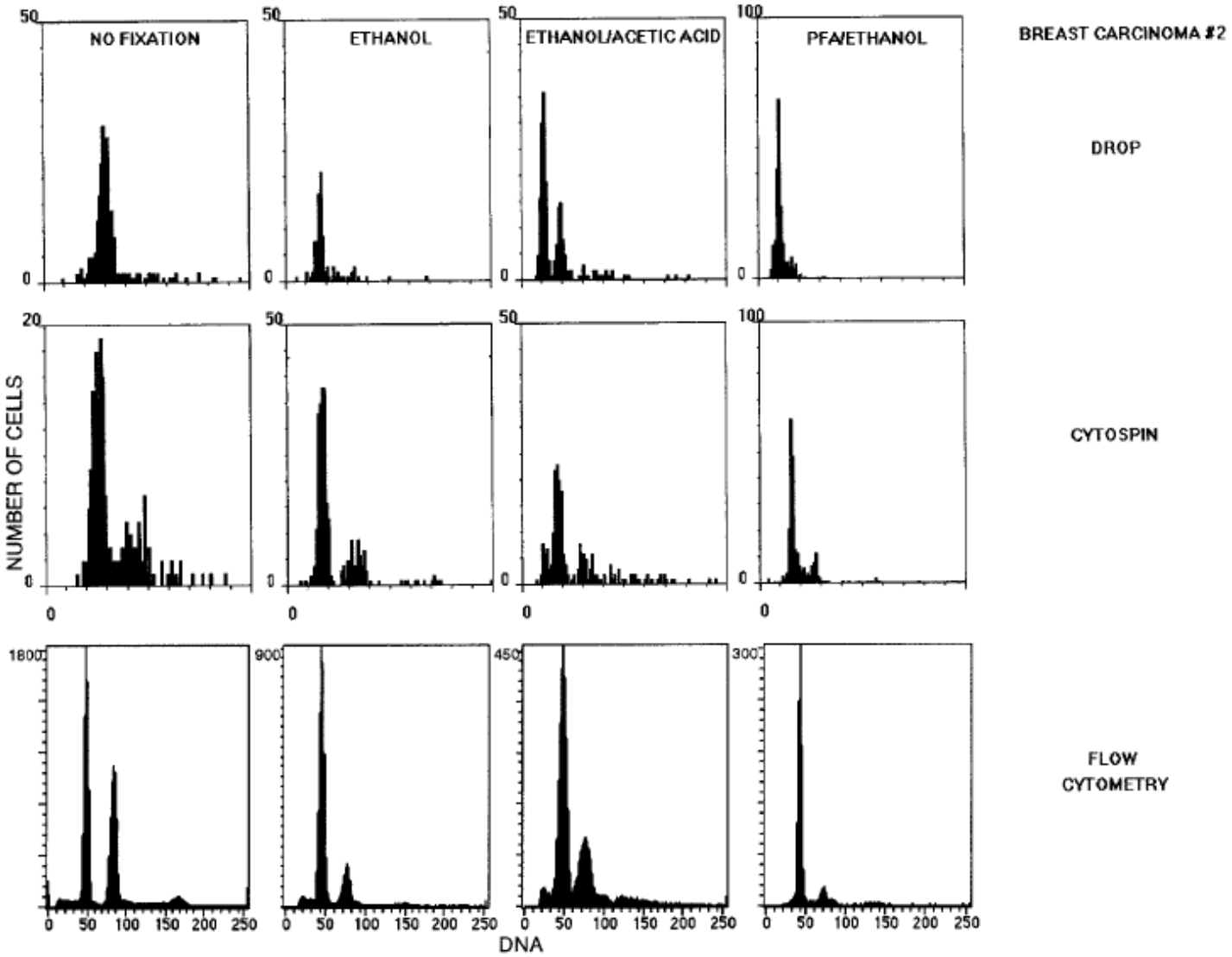


FIG. 2

Corresponding images of DAPI stained cells

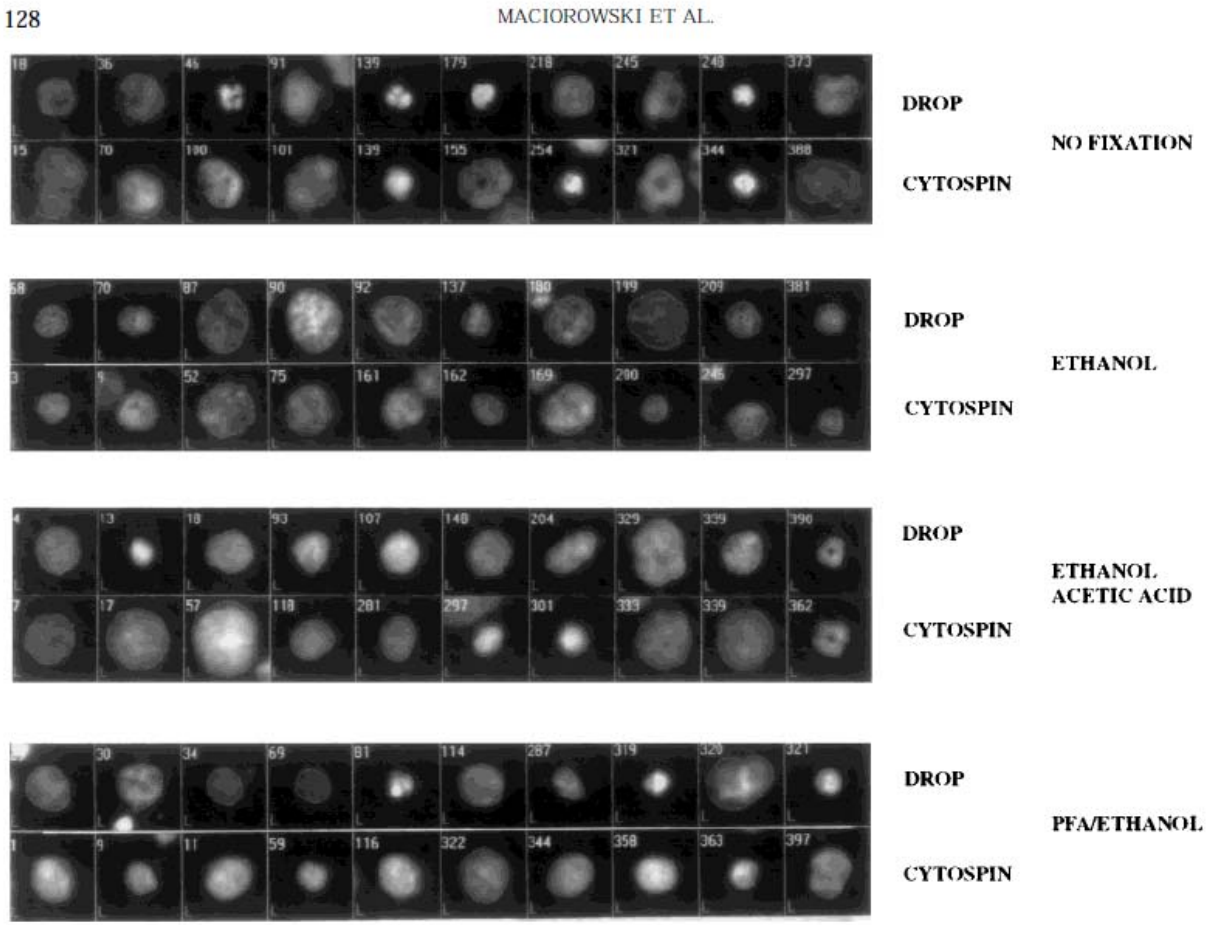


FIG. 3. Examples of nuclear chromatin morphology for drop and cytospin preparations of unfixed, ethanol, ethanol/acetic acid, and paraformaldehyde/ethanol fixed breast tumor cells.

Guidelines for Improving the Reproducibility of Quantitative Multiparameter Immunofluorescence Measurement by Laser Scanning Cytometry on Fixed Cell Suspensions from Human Solid Tumors
Shackney et al Cytometry part B(Clinical Cytometry)70B:10-19 (2005)

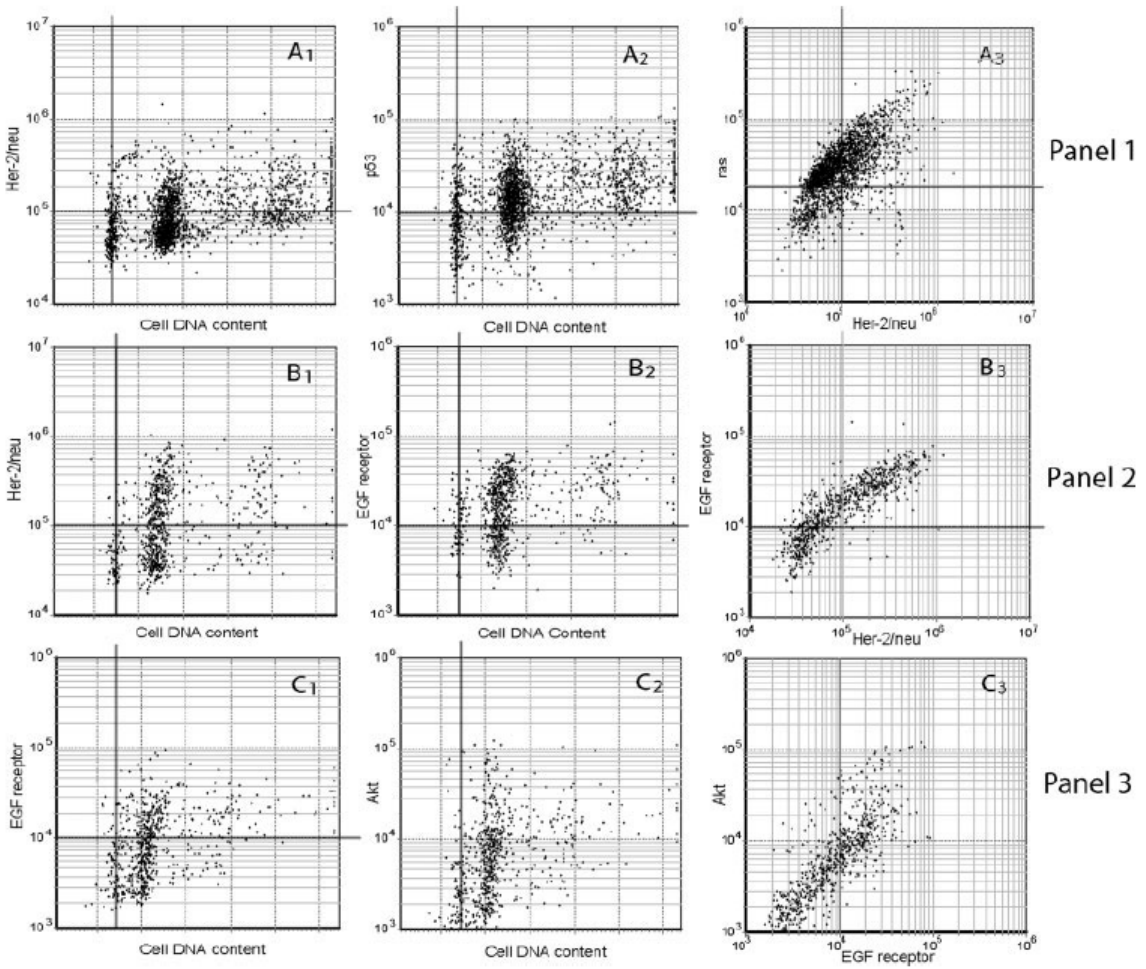
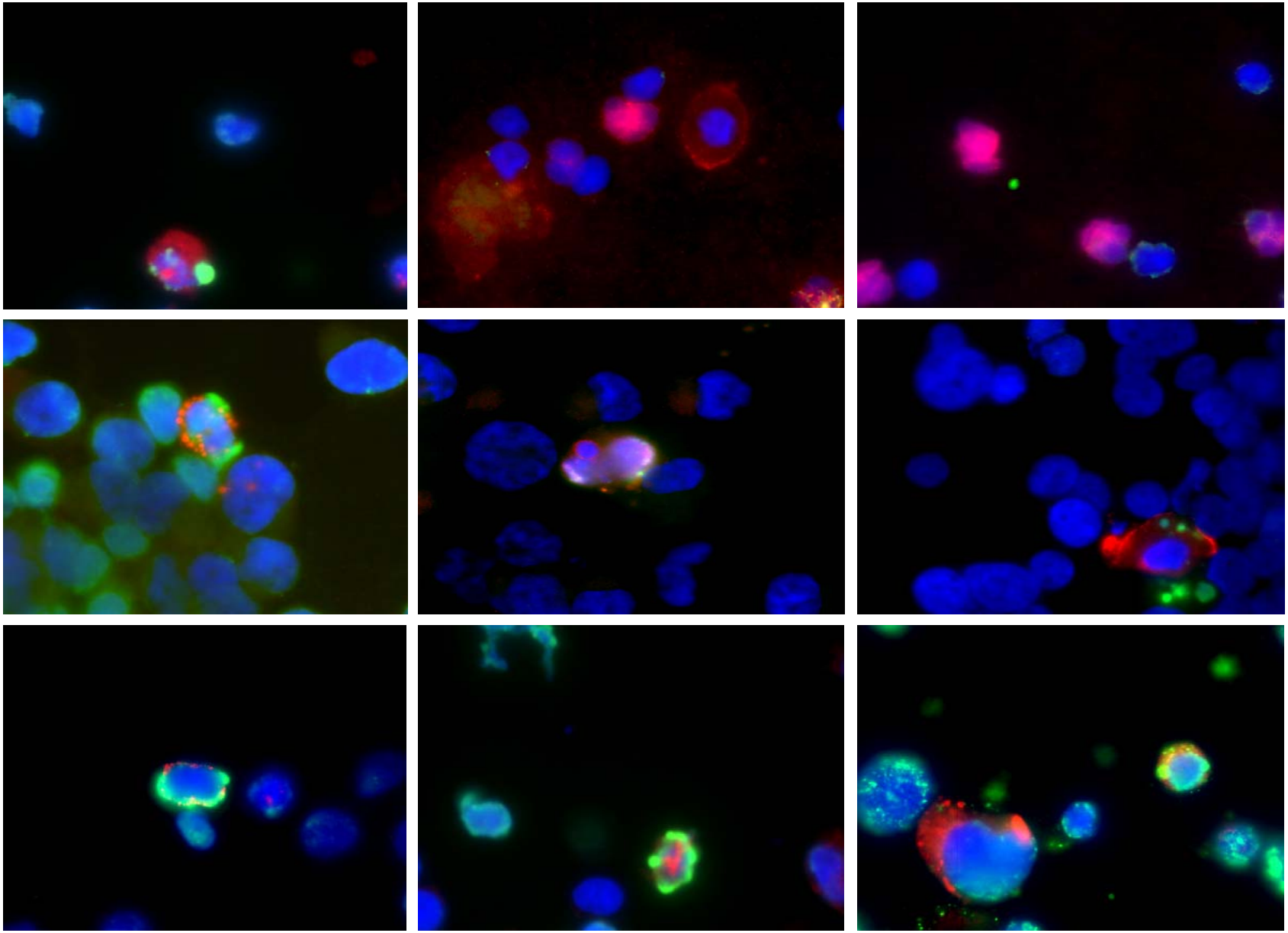


FIG. 4. Selected two-parameter dot plots of data from linked multicolor panels of immunofluorescence measurements obtained from a sample of human primary breast cancer. (A_1–A_3) Data from panel 1: cell DNA content, Her-2/neu, p53 protein, and ras protein. (B_1–B_3) Data from panel 2: cell DNA content, Her-2/neu, EGF receptor, and erbB-3. (C_1–C_3) Data from panel 3: cell DNA content, EGF receptor, Akt protein, and Erk1/2. For discussion, see text. In panels with cell DNA content on the abscissa, vertical reference lines indicate diploidy. In other panels, vertical and horizontal lines indicate cutoffs between normal and abnormal levels, when appropriate. Data were corrected for cell aggregates, autofluorescence, and cross-talk.



Different patterns of staining seen in breast tumor FNA:
anti-active caspase-3 (red) , TUNEL (green), and DAPI (blue)

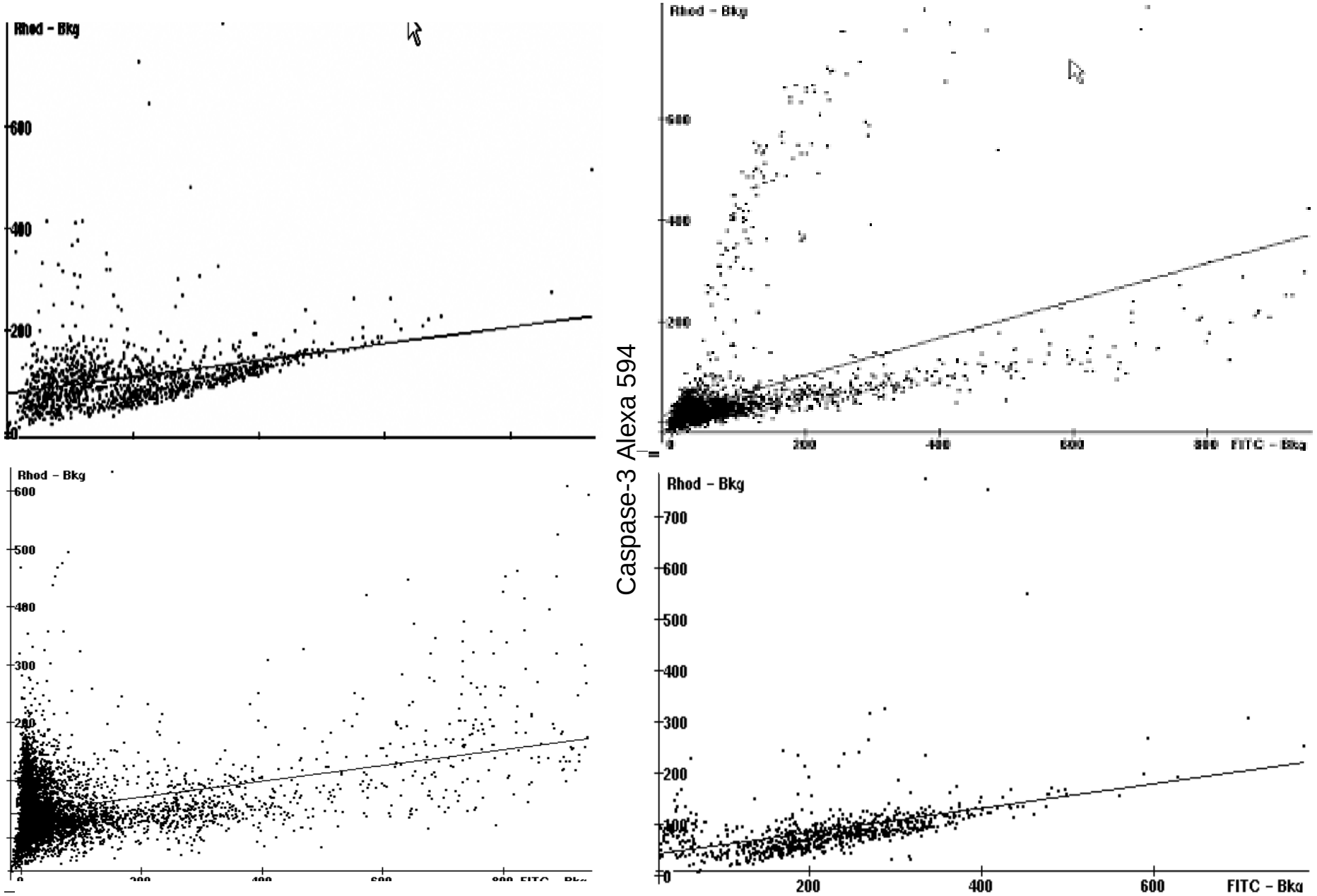
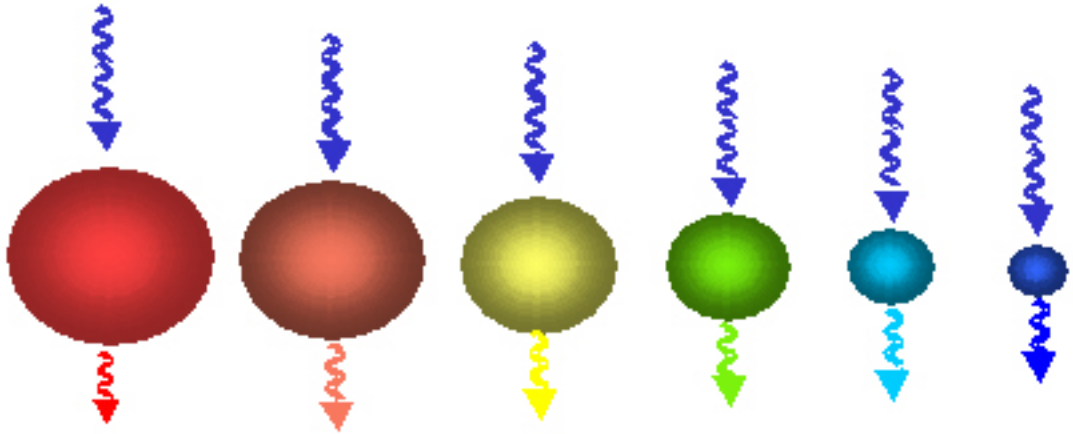
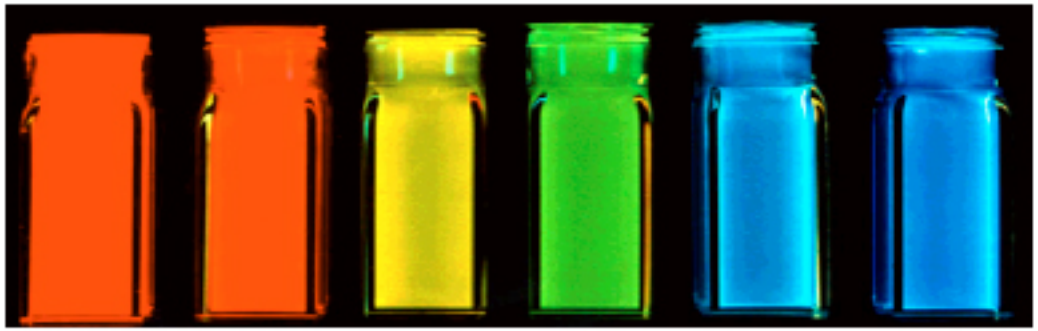


Image analysis scattergrams of breast tumor cells stained with anti-active caspase-3 (red) vs TUNEL (green)

Quantum dot technology

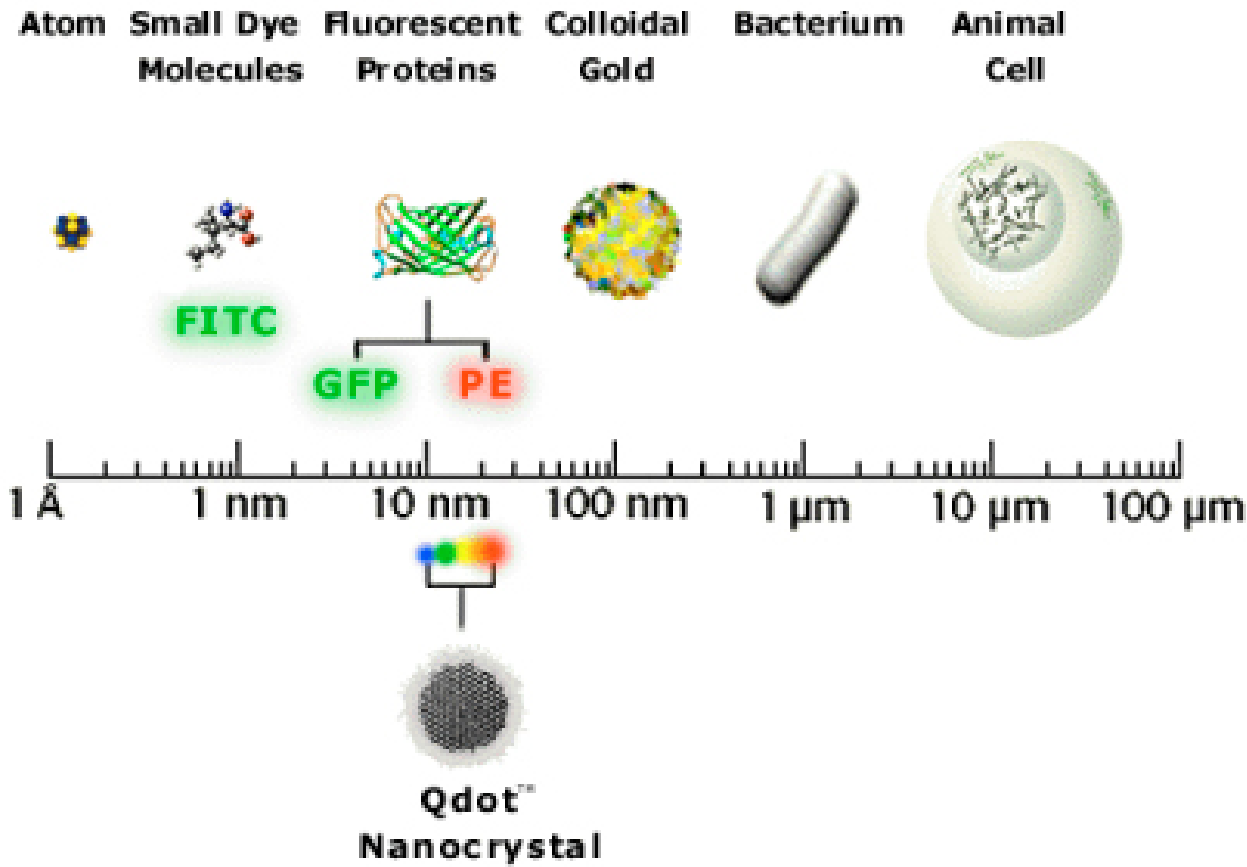


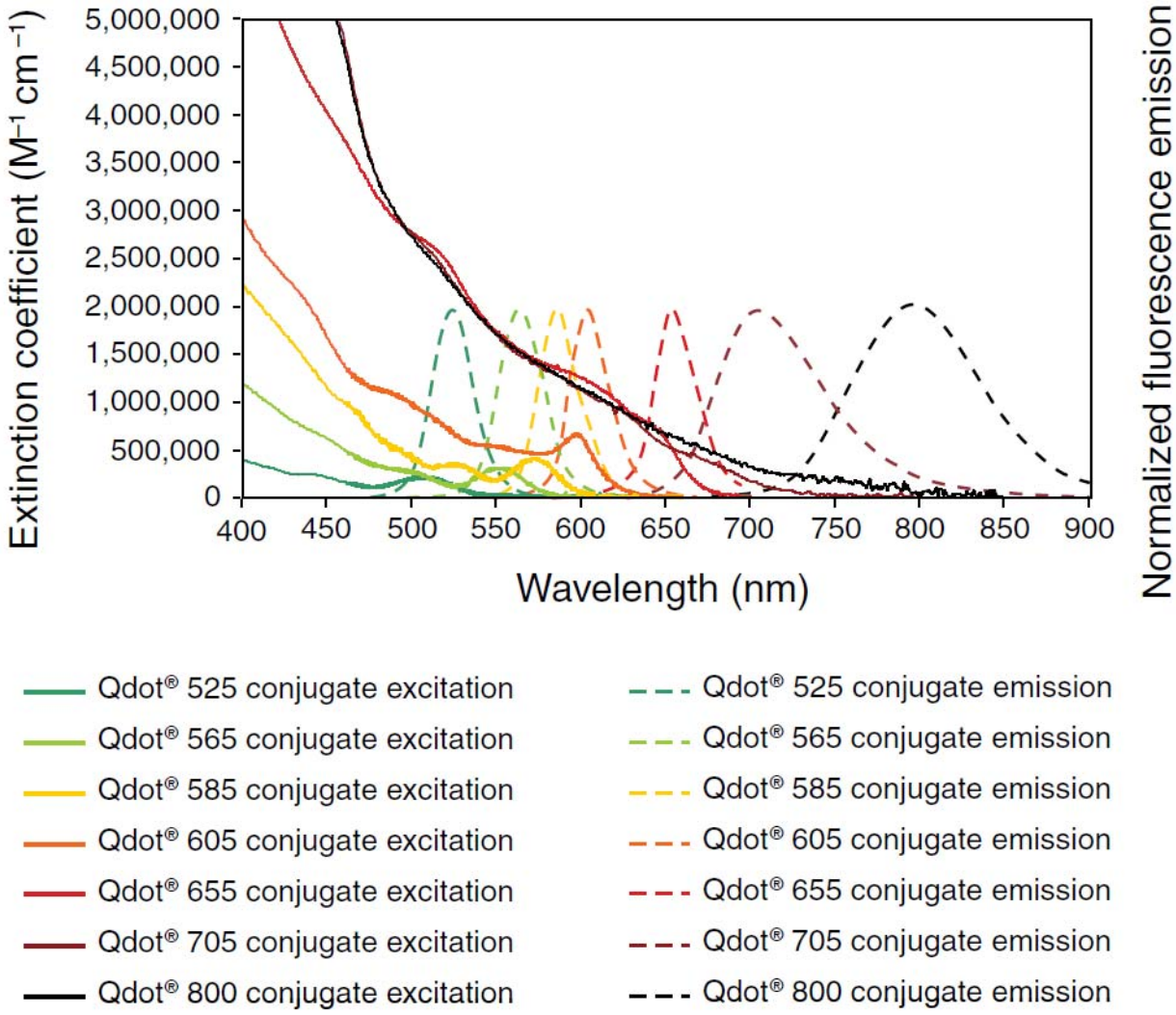
Nanocrystals absorb light then re-emit the light in a different color – the size of the nanocrystal (at the Angstrom scale) determines the color



Felice Frankel

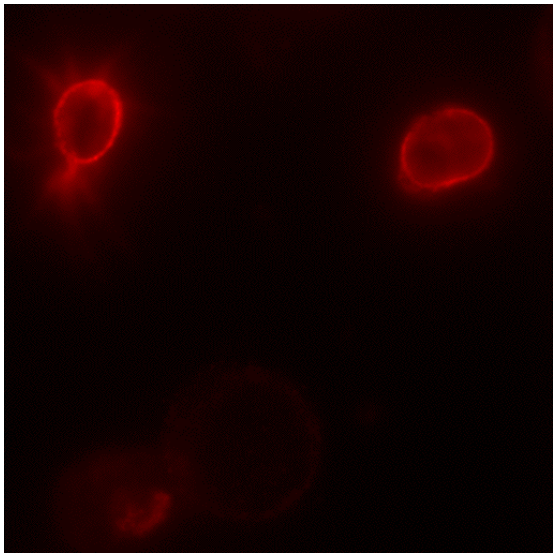
Six different quantum dot solutions are shown excited with a long wave UV lamp


Atom
Small Dye Molecules
Fluorescent Proteins
Colloidal Gold
Bacterium
Animal Cell
FITC
GFP
PE
1 Å
1 nm
10 nm
100 nm
1 µm
10 µm
100 µm
Qdot™ Nanocrystal


Extinction coefficient (M⁻¹ cm⁻¹)
5,000,000
4,500,000
4,000,000
3,500,000
3,000,000
2,500,000
2,000,000
1,500,000
1,000,000
500,000
0
400
450
500
550
600
650
700
750
800
850
900
Wavelength (nm)
Normalized fluorescence emission
Qdot® 525 conjugate excitation
Qdot® 565 conjugate excitation
Qdot® 585 conjugate excitation
Qdot® 605 conjugate excitation
Qdot® 655 conjugate excitation
Qdot® 705 conjugate excitation
Qdot® 800 conjugate excitation
Qdot® 525 conjugate emission
Qdot® 565 conjugate emission
Qdot® 585 conjugate emission
Qdot® 605 conjugate emission
Qdot® 655 conjugate emission
Qdot® 705 conjugate emission
Qdot® 800 conjugate emission

Qdot double staining

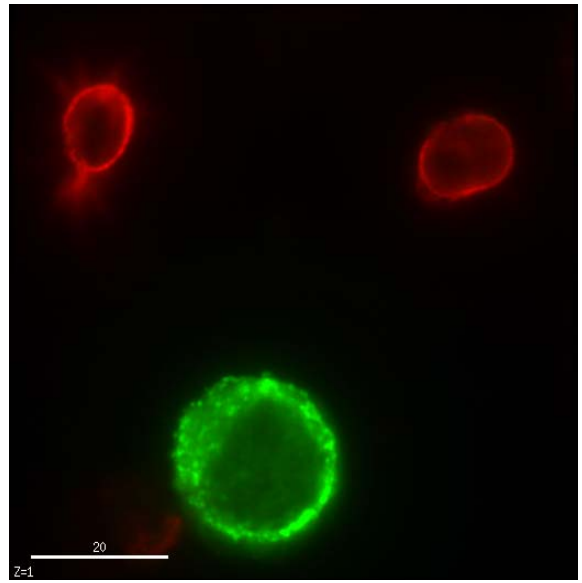
Excitation filter 460

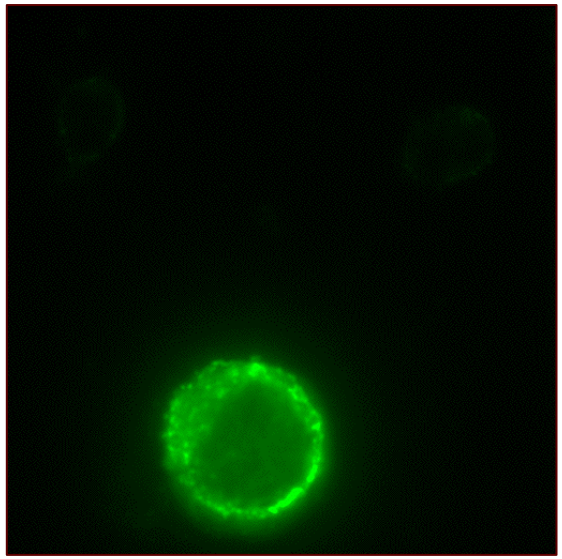


Cytospin: Lymphocytes +melanoma

QD605 anti CD45
QD655 streptavidin+ biot anti melan A + HMB45

Emission filter 605/20





Emission filter 655/20

Qdots vs classic fluorochromes

Advantages

- Narrow band emission: no spectral overlap
- bright
- Much more photostable than classic fluorochromes

Disadvantages

- Standardized protocols not yet in place
- Range of antibodies coupled directly to Qdots on the market limited

Multiparameter Qdot review articles

Quantum Dots light up Pathology

Tholouli, Sweeney, Barrow, Clay, Hoyland and Byers

J. Pathology 2008; 216:275-285

Bioconjugated Quantum Dots for Multiplexed and Quantitative Immunohistochemistry

Xing, Chaudry, Shen, Kong, Zhau, Chung, Petros, O'Regan, Yezhelyev, Simmons, Wang and Nie

Nature Protocols, vol.2 no.5, 1152-1165, 2007

Solid tumor multicolor cytometry

- Solid tumors better adapted to slide based assays
  - No loss of cells
  - No loss of clumps containing tumor cells
  - New tools available allow better and more stable multicolor staining
  - Potential for classical morphological assessment by pathologist