Applications of Flow Cytometry in Diagnostic Cytology of Body Cavity Fluids

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Jackson Memorial Medical Center
Univ. of Miami Miller School of Medicine

JACKSON MEMORIAL MEDICAL CENTER
UNIVERSITY OF MIAMI MILLER SCHOOL OF MEDICINE

Diagnostic Cytology of Cells in Body Cavity Fluids

- **Pleural or peritoneal fluids are often present in patients with lung, breast and ovarian tumors.**
- **AT UM/JMH more than 27,000 body cavity fluid specimens are processed annually.**
- **30-50% of body cavity fluids from patients with a proven malignancy are false negative as diagnostic cytology can not find tumor cells.**

Motherby et al., Diag.Cytopath.20: 350, 1999
Ganjei et al., Acta Cytologica, 48: 653, 2004

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False Negative in Diagnostic Cytology of Body Cavity Fluids

- Tumor cells may not be present in peritoneal or pleural fluid.
- Enough tumor cells may not be present for visual examination under a microscope.
- Tumor cells may be morphologically indistinguishable from normal epithelial and mesenchymal cells.

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Diagnostic Cytology of Body Cavity Fluids

- Cellular patterns and morphological characteristics of the individual cells.
- In samples with “atypical cells”, immunocytochemistry may be used to identify tumor cells and suggest the possible site of origin.

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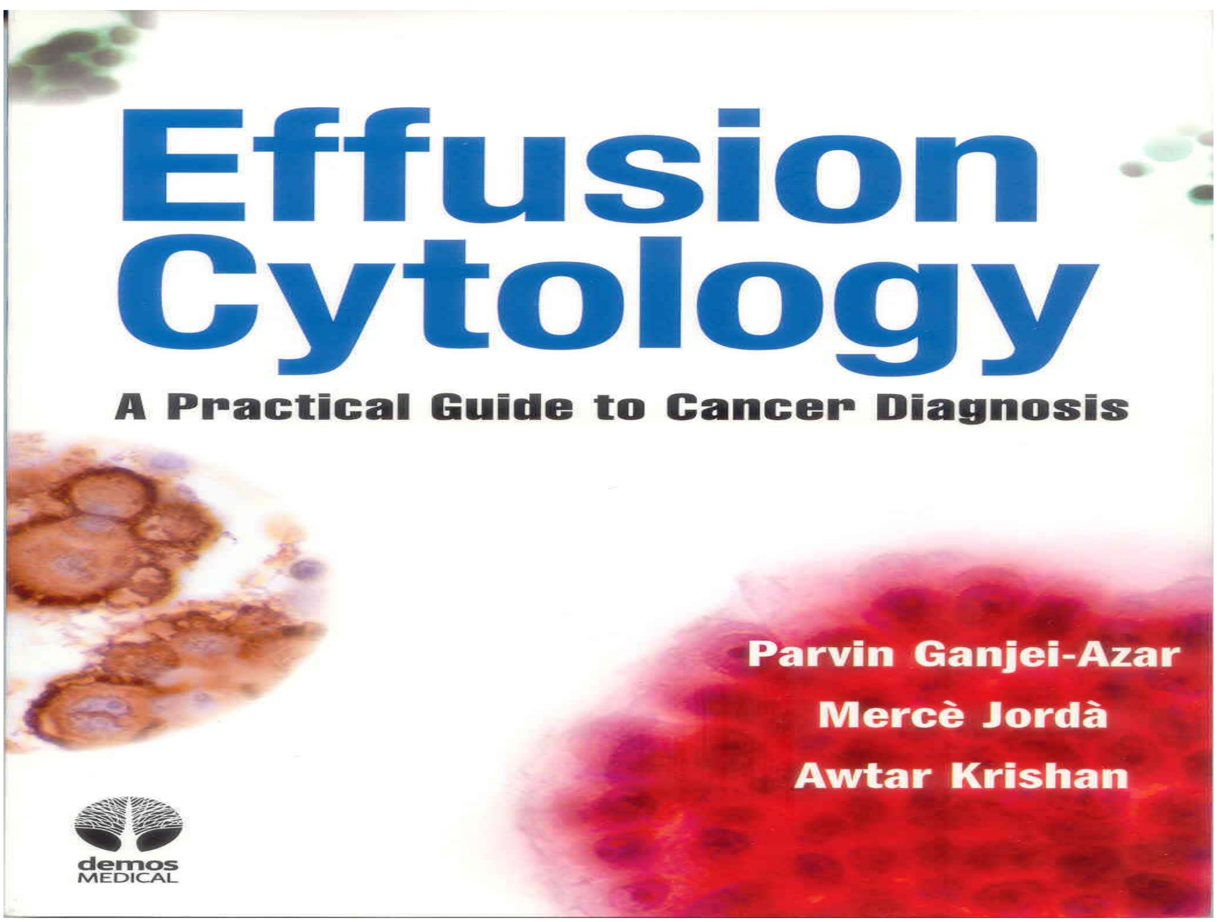
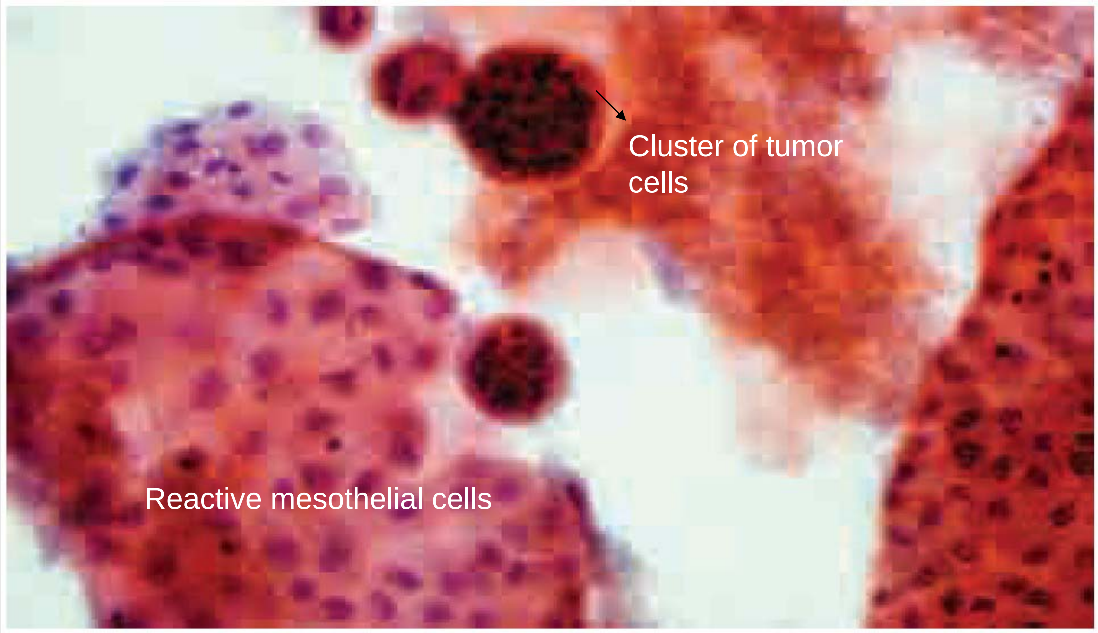


Tight cluster of malignant cells in pleural fluid of a breast ca.



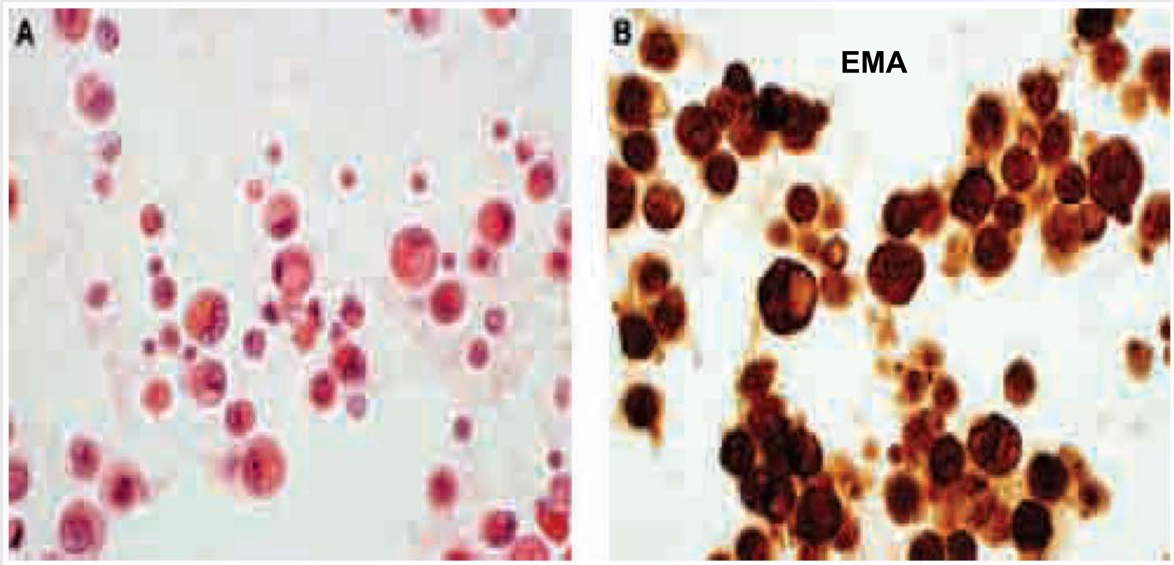
Ganjei, Jorda & Krishan
Effusion Cytology, Demoss Medical

Ber-EP4/EMA

- **Epithelial Membrane Antigen.**
- **Expressed in:**
  75-90 % of carcinomas,
  4% of mesotheliomas
  0% of benign mesothelial proliferation.

Comin CE, et el. Amer. J. Surg. Path. 31:1139-48, 2007.
Davidson B, et al., Diagn Cytopathol. 35: 568-78, 2007.

EMA positive cells in peritoneal fluid of a gastric ca.



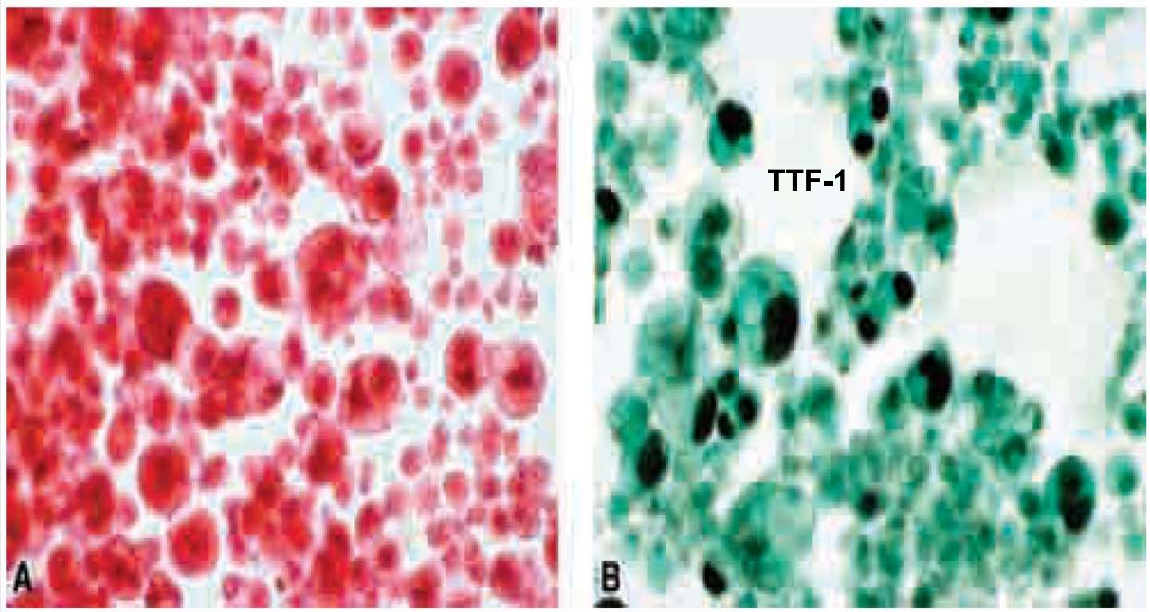
Ganjei, Jorda & Krishan
Effusion Cytology, Demoss Medical

Thyroid Transcription Factor-1

- **A nuclear receptor found in 90% small-cell lung adenocarcinomas, ~23% of endometrial and endocervical ca. with negligible expression in squamous cell carcinomas.**

- **Siami, K., et al. Am J Surg Pathol. 31: 1759-63, 2007.**
- **Kalhor, N., et el. Mod. Path., 19: 117-23, 2006.**
- **Ordonez, NG, Mod Path. 19: 417-28, 2006.**

TTF-1 Positive cells in pleural fluid of an adenocarcinoma



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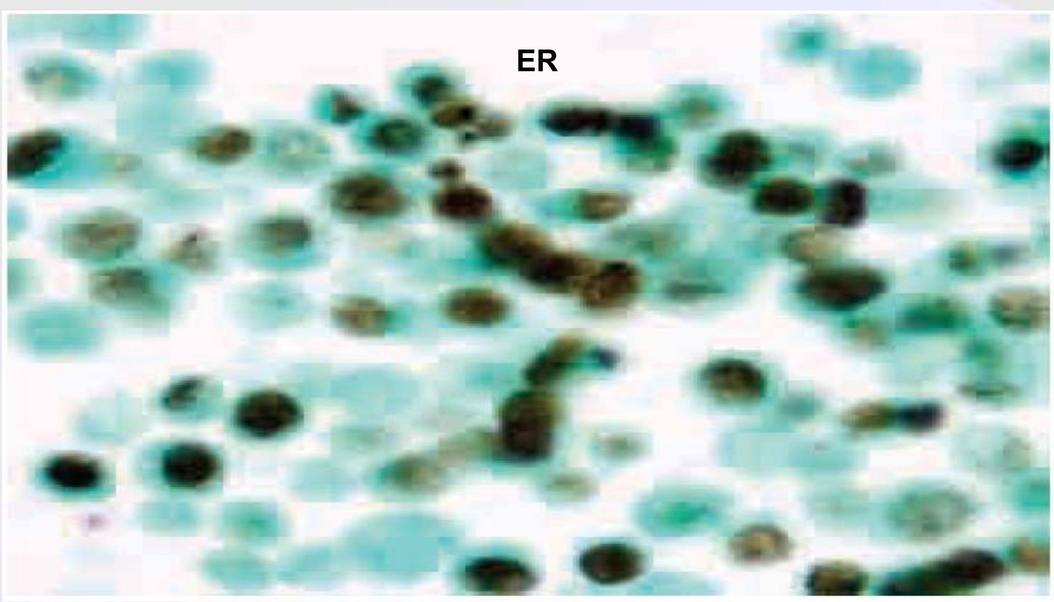
ER positive cells in pleural fluid of a Breast CA



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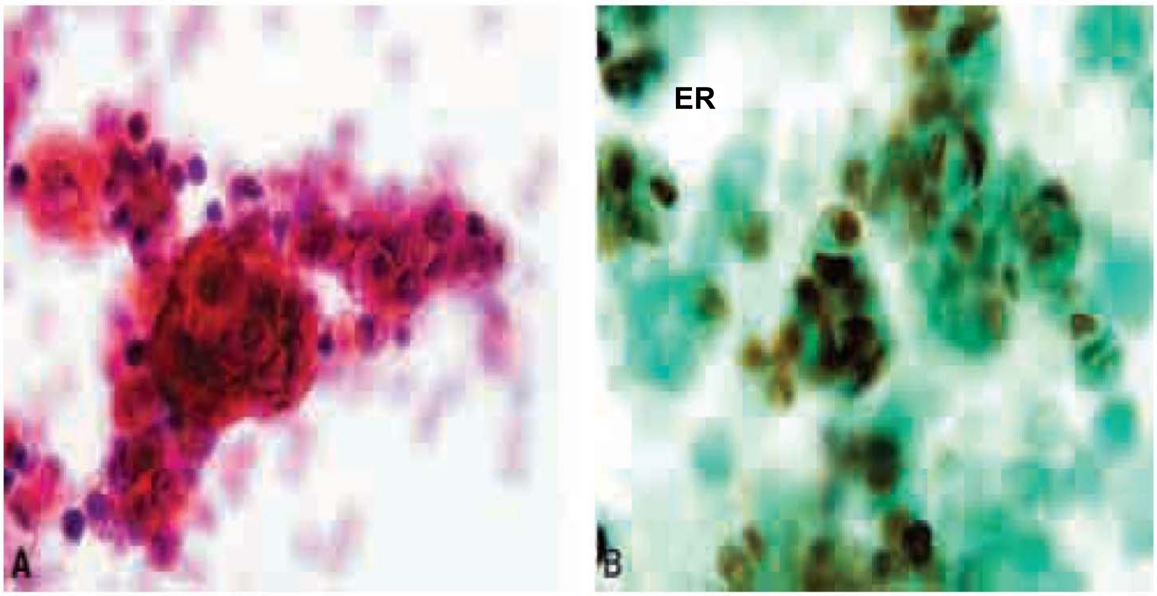
ER positive cells in pleural fluid of a breast ca.



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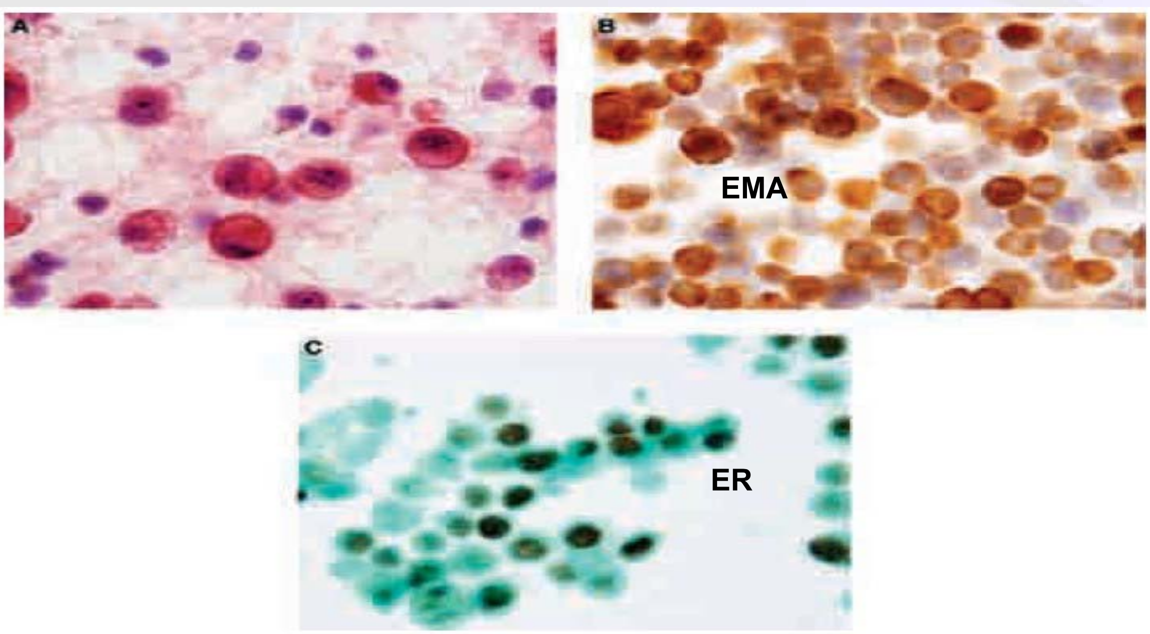
EMA and ER positive cells in pleural fluid of a breast ca.



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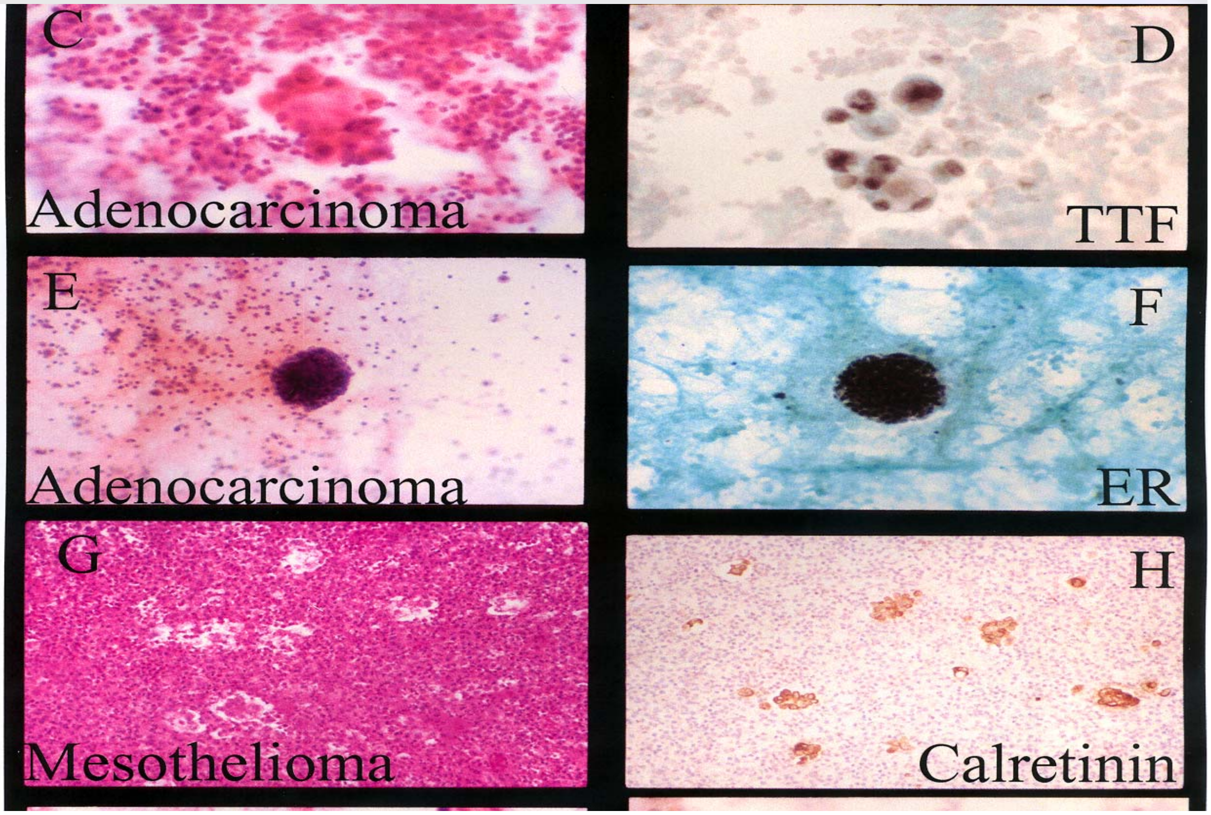
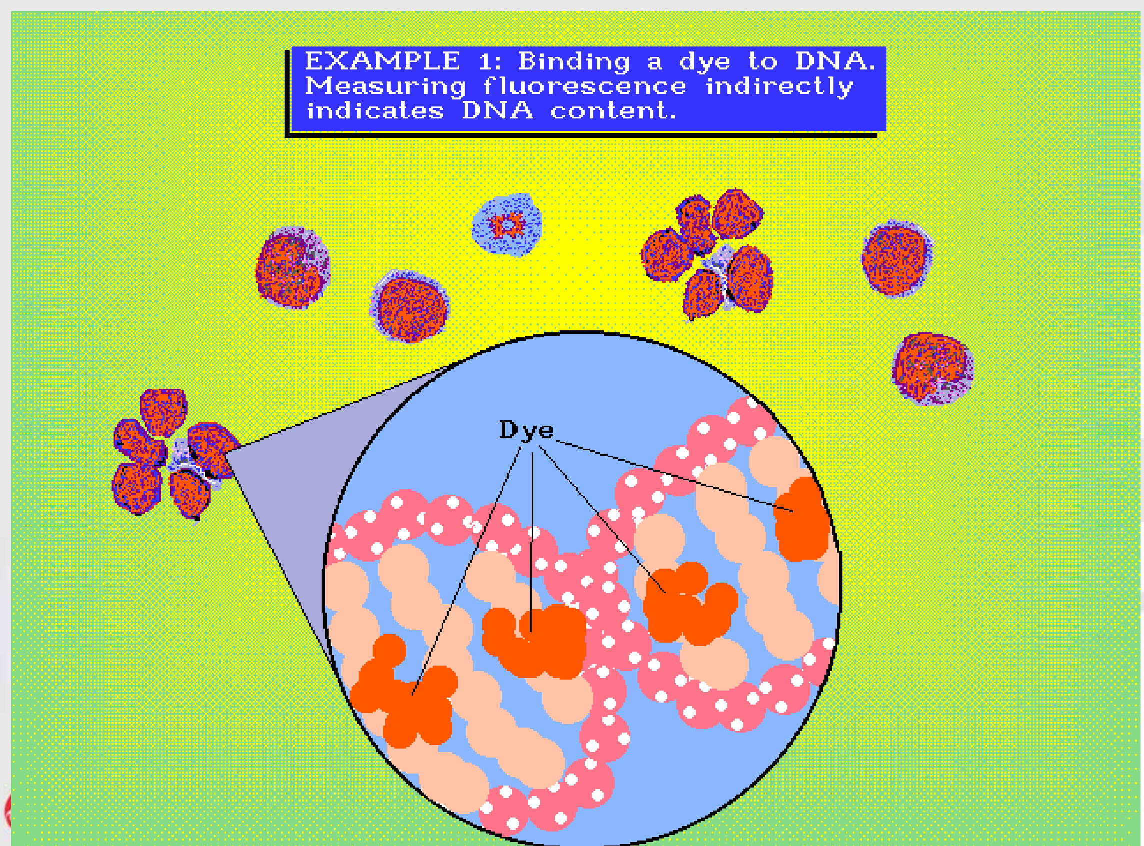
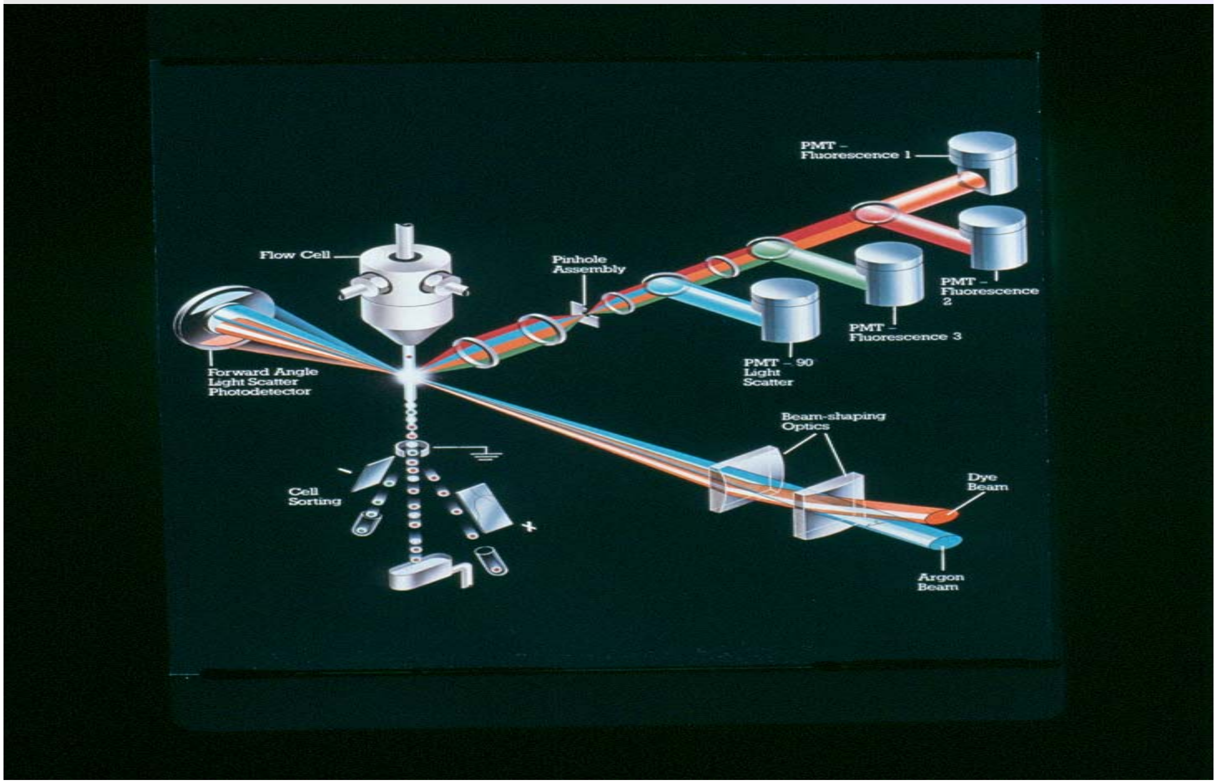
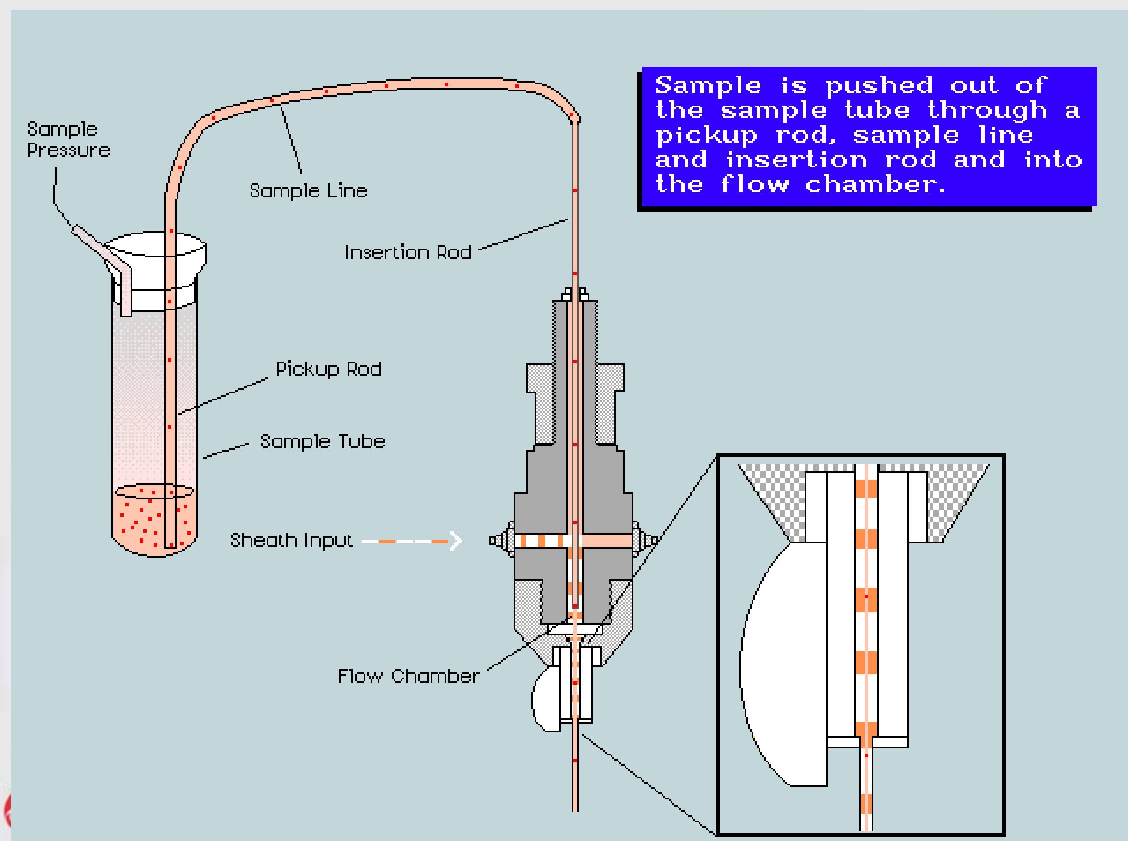
Marker Expression of TTF-1, ER & Calretinin

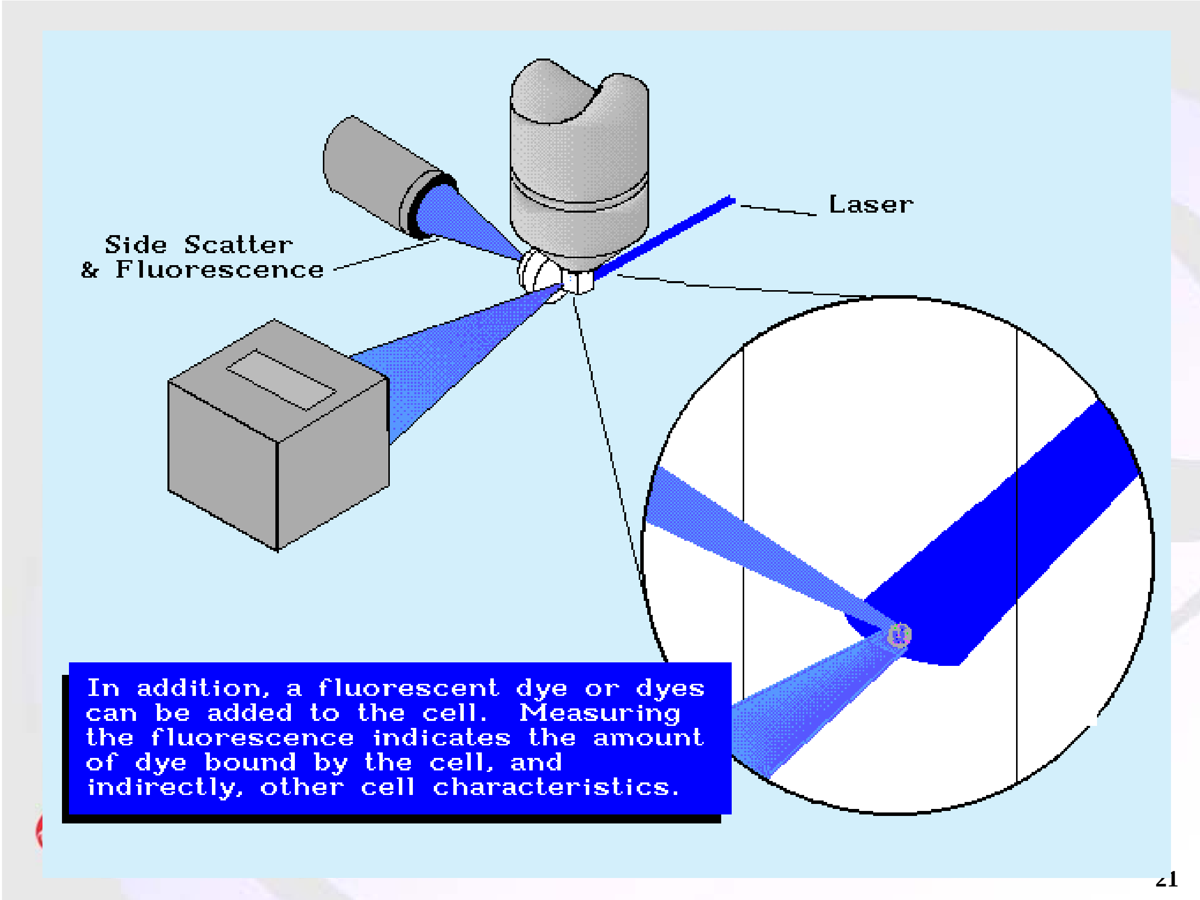


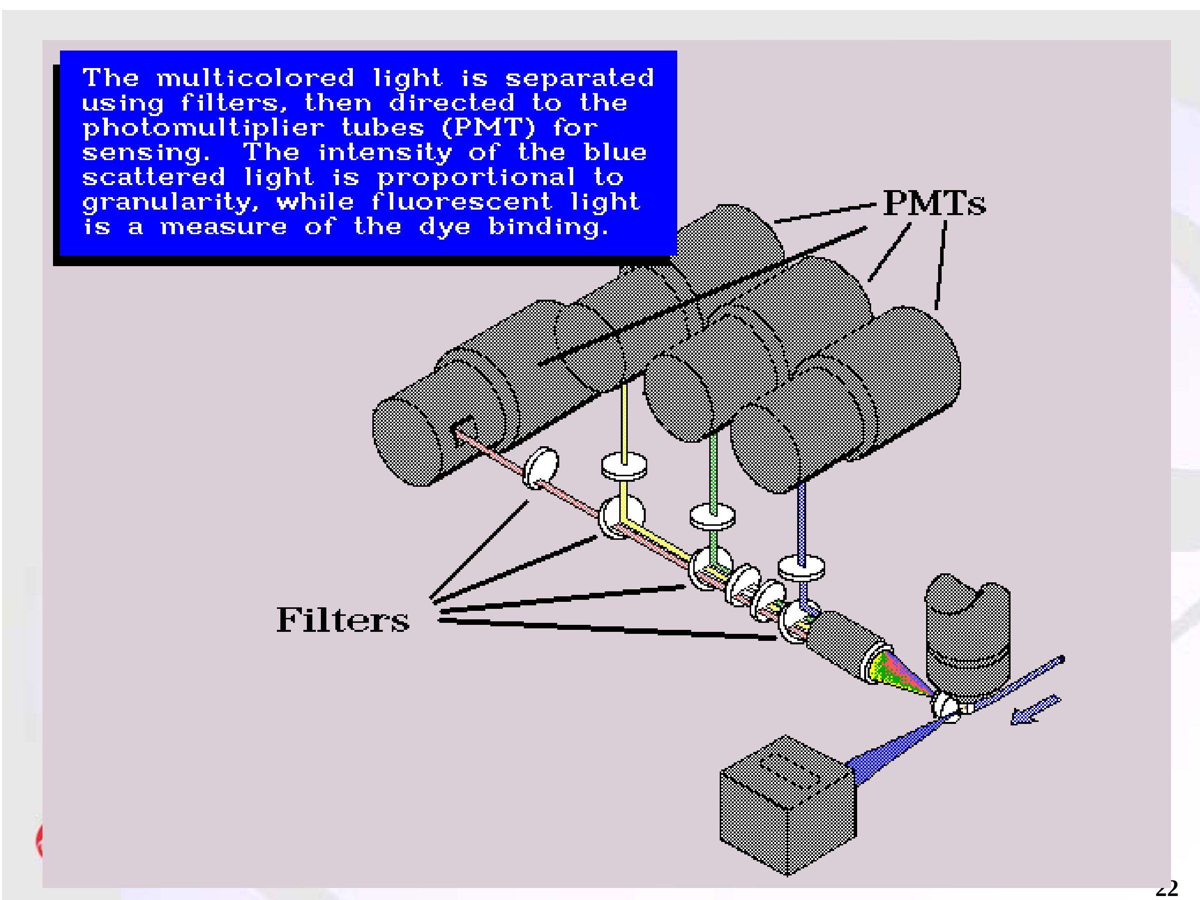


BASICS OF A FLOW CYTOMETER



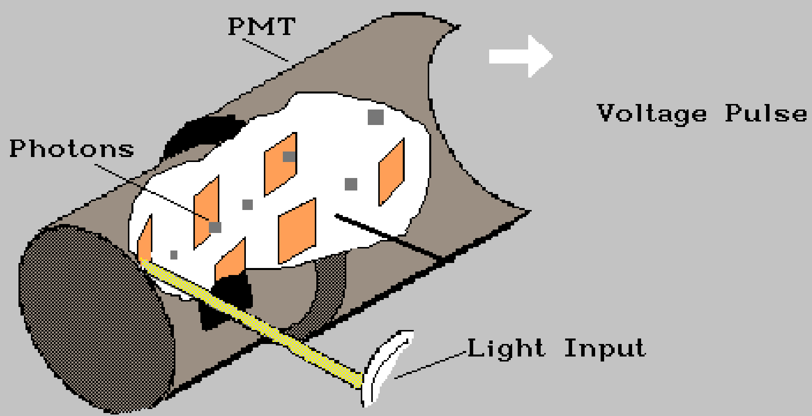



Laser
Side Scatter
& Fluorescence
In addition, a fluorescent dye or dyes can be added to the cell. Measuring the fluorescence indicates the amount of dye bound by the cell, and indirectly, other cell characteristics.

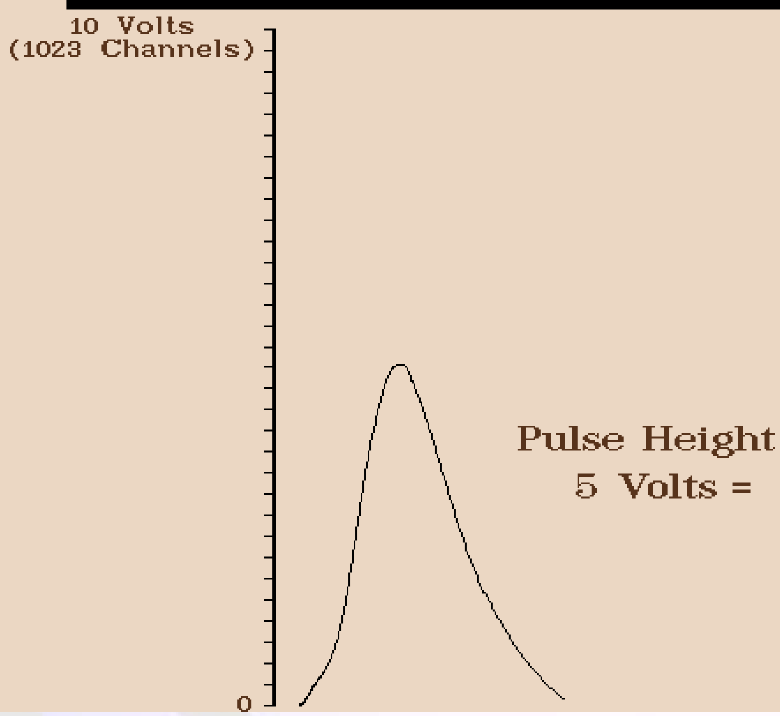

The multicolored light is separated using filters, then directed to the photomultiplier tubes (PMT) for sensing. The intensity of the blue scattered light is proportional to granularity, while fluorescent light is a measure of the dye binding.
PMTs
Filters

Conversion of Light to a Voltage Pulse

The PMT converts the light to a voltage pulse which rises and falls with the amount of light entering it.

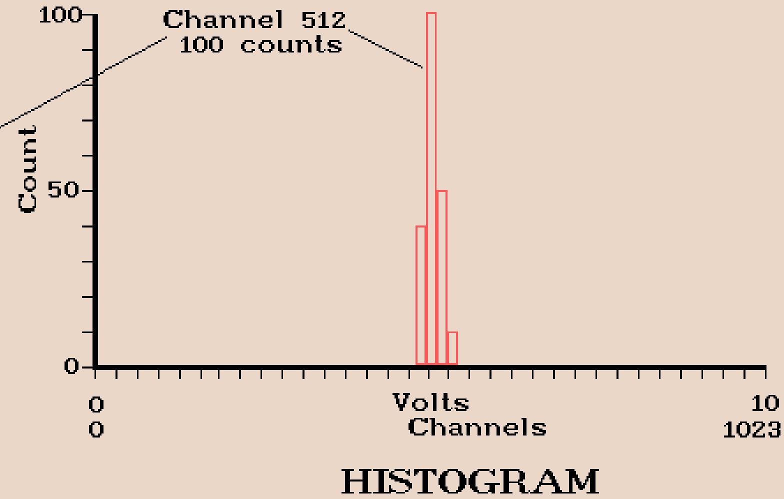


The 0 to 10 volt pulse heights are measured in increments called channels (0 to 1023). A 5 volt pulse would be at the 512 channel position. This process is called Analog to Digital Conversion.

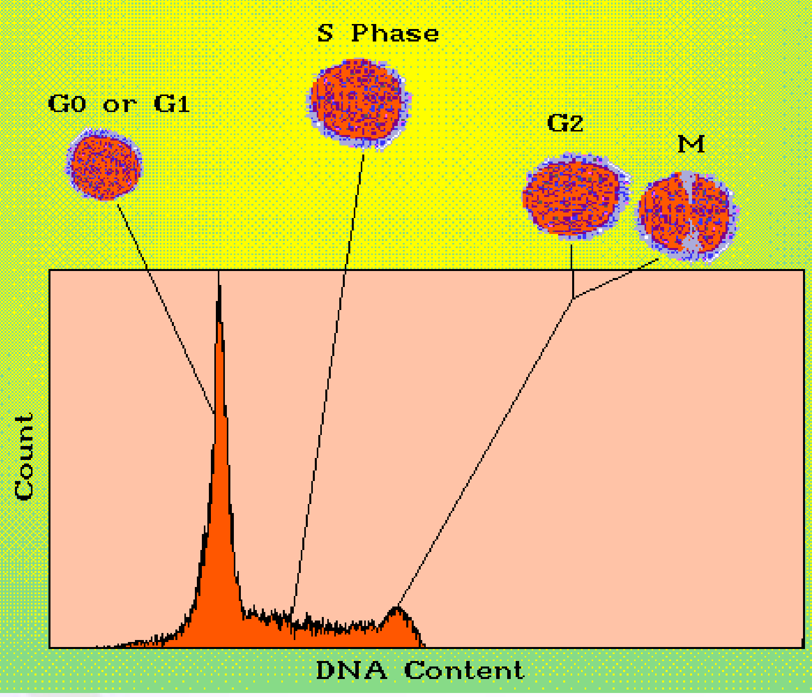


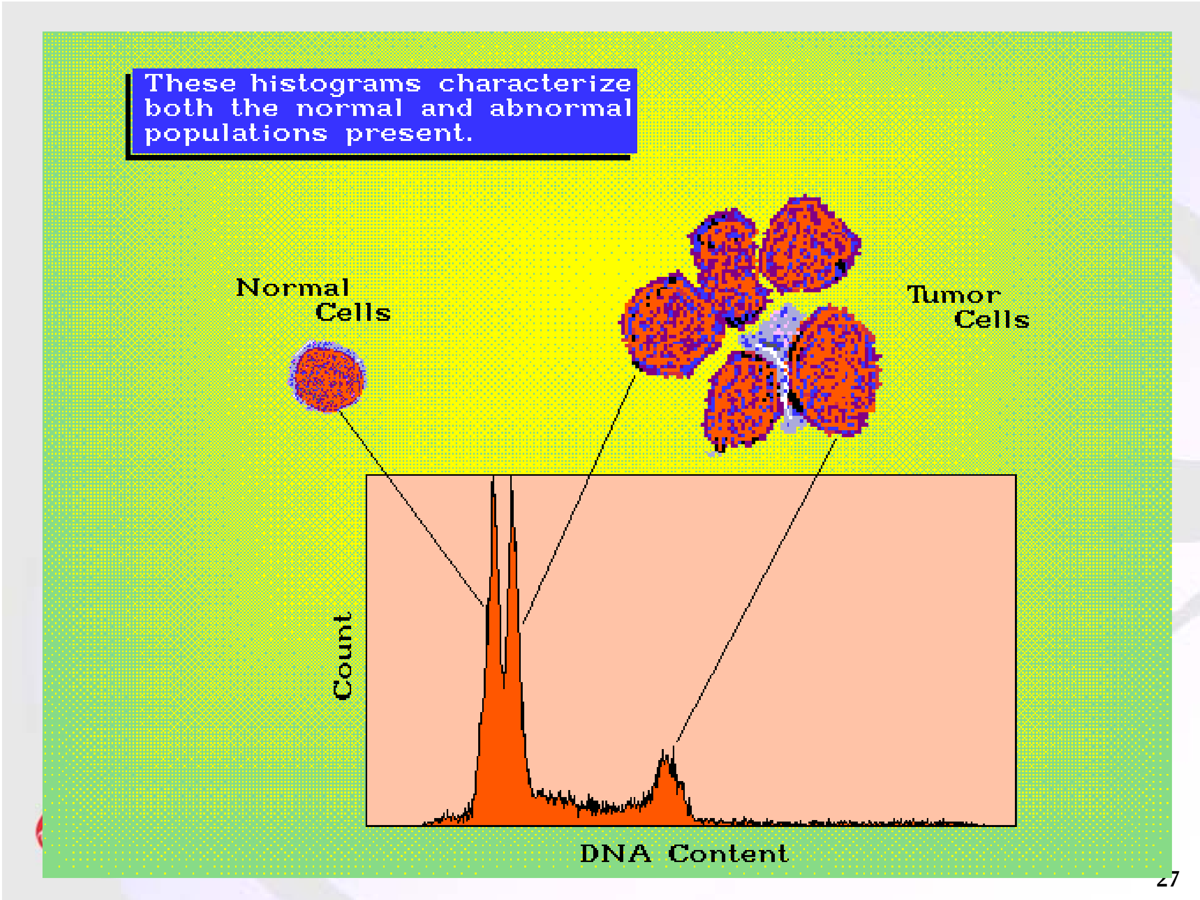
These converted pulses increment counters for each channel. A plot of the counts versus the channels is called a histogram.

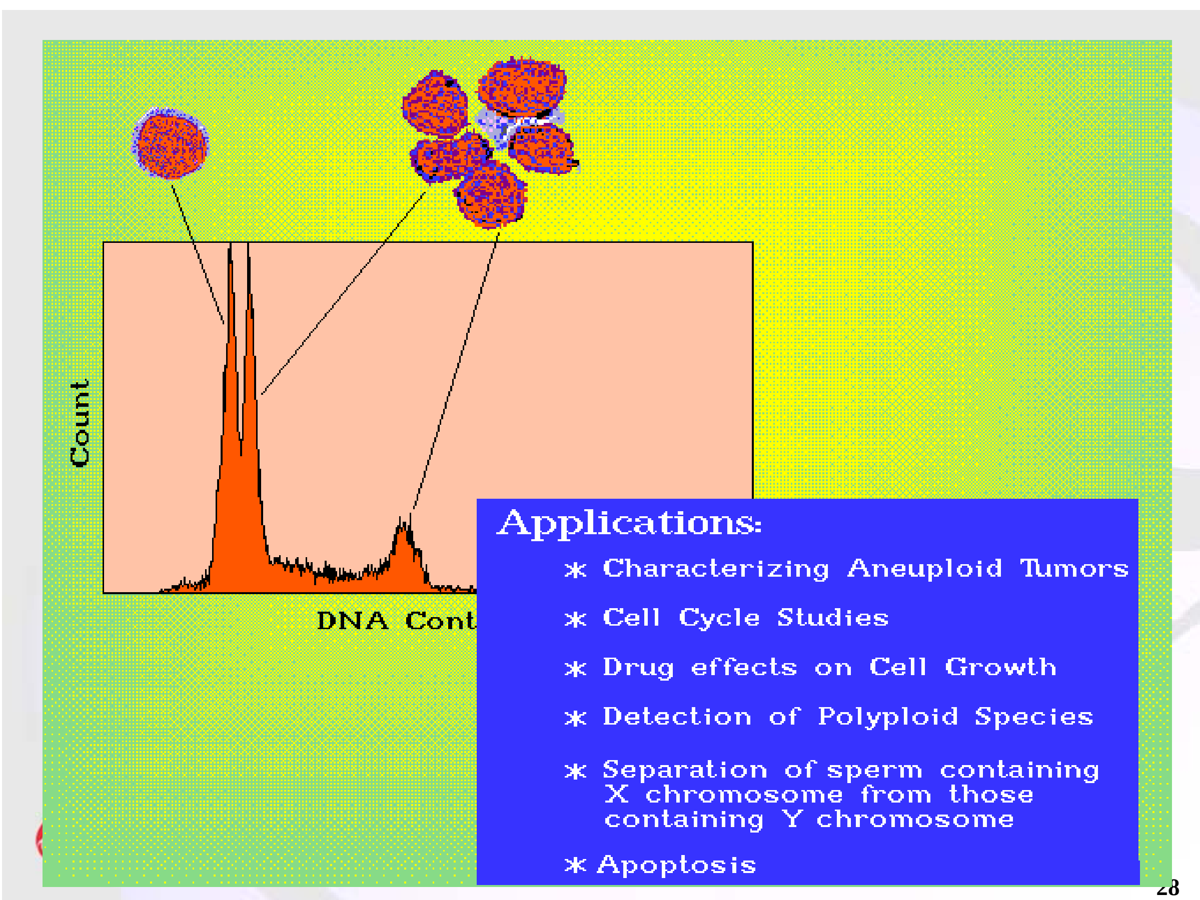
Channel	Count
510	0
511	40
512	100
513	50
514	10
515	0



The number of cells with varying amounts of DNA can be displayed and analyzed.




These histograms characterize both the normal and abnormal populations present.
Normal Cells
Tumor Cells
Count
DNA Content
27


Count
DNA Cont
Applications:
⁎ Characterizing Aneuploid Tumors
⁎ Cell Cycle Studies
⁎ Drug effects on Cell Growth
⁎ Detection of Polyploid Species
⁎ Separation of sperm containing X chromosome from those containing Y chromosome
⁎ Apoptosis
28

Flow cytometric Analysis of Body Cavity Fluids

- Flow cytometry was extensively used in 70's for the detection of malignant cells with aneuploid DNA content in peritoneal, pleural, and cerebrospinal fluids.
- In several studies, flow analysis detected cells with aneuploid DNA content in fluids with "negative cytology".
- On re-examination these samples were found to contain tumor cells thus reducing the false-negative rate from 21.8% to 4.7%.
-

Lovecchio, et al.,Obstet.Gynecol.67: 675, 1986

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Nuclear Volume/protein content vs. DNA Content of Human Tumor Cells

- As tumor cells and nuclei are often larger in size than normal cells, flow cytometric analysis of nuclear volume/protein content vs. DNA content could be used to differentiate between normal and tumor cells.
- Expression of secondary markers could then be studied in tumor nuclei to suggest the site of their origin.

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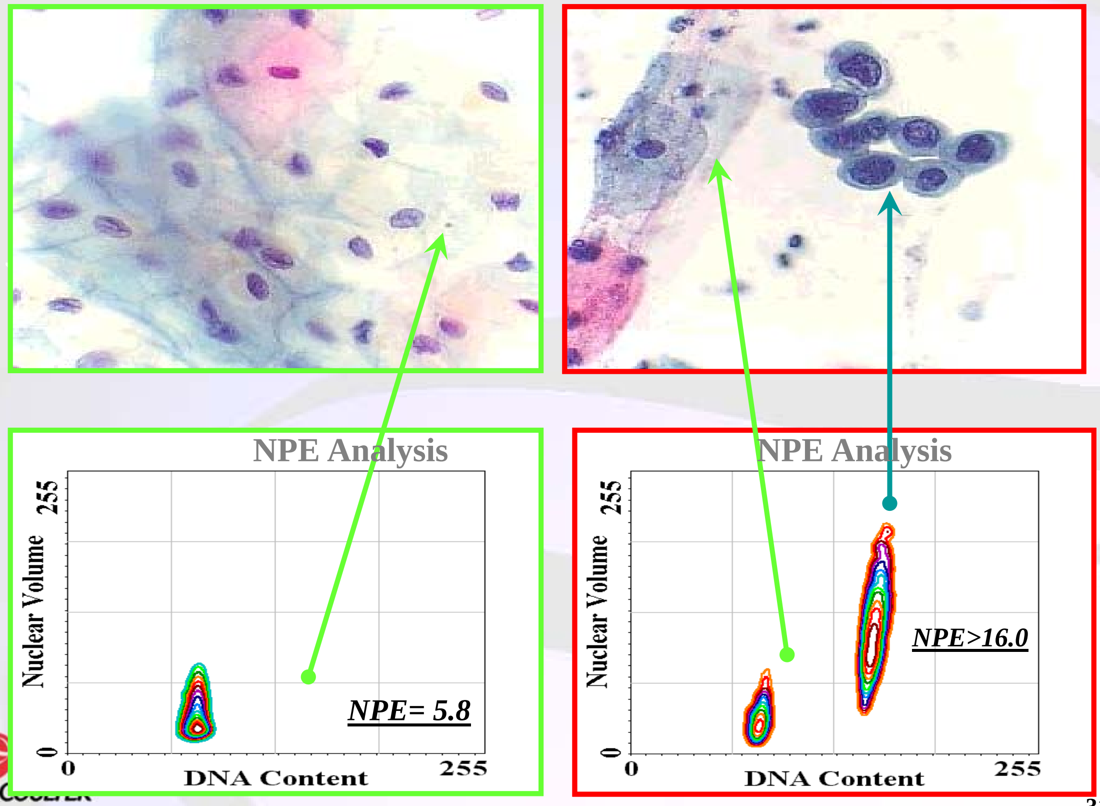
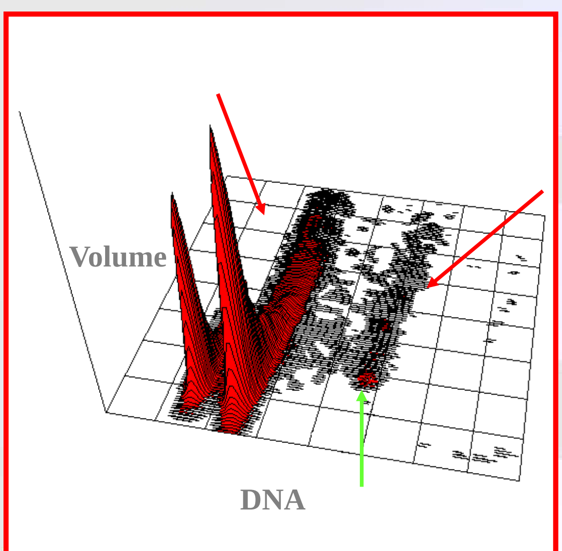
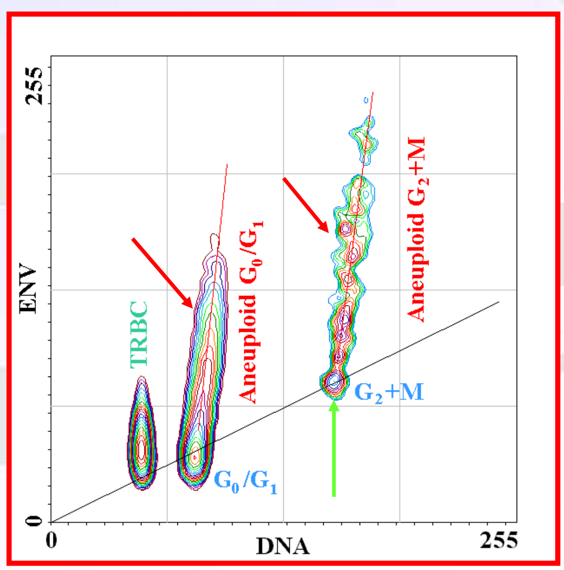
Normal Nuclei / Malignant Nuclei



Nuclear Volume vs. DNA Content

Prostate Cancer

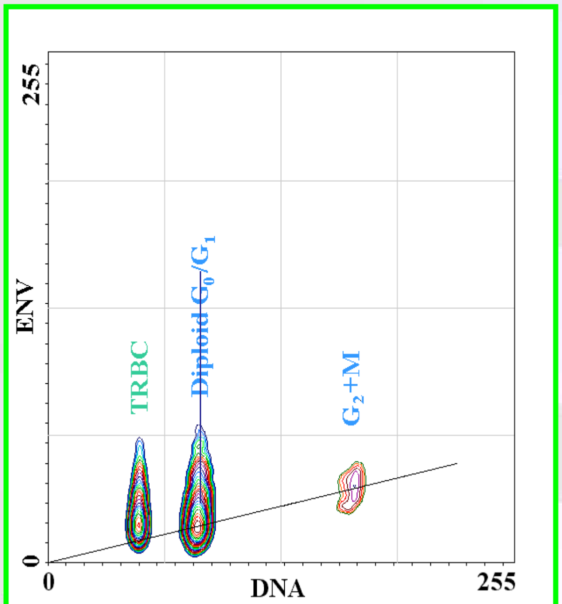




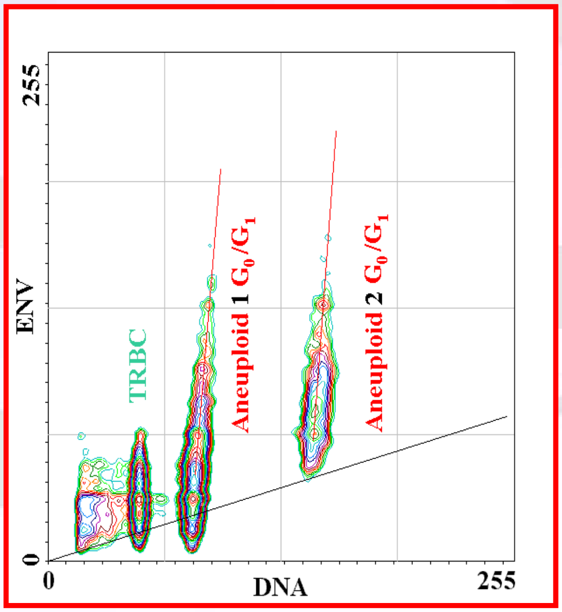
Krishan, et al. Cytometry 43, 2001

Nuclear Volume vs. DNA Content

Normal Breast



Primary Breast Cancer



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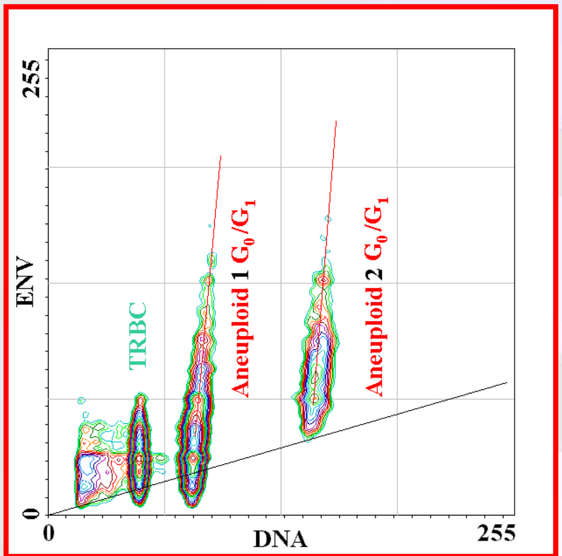
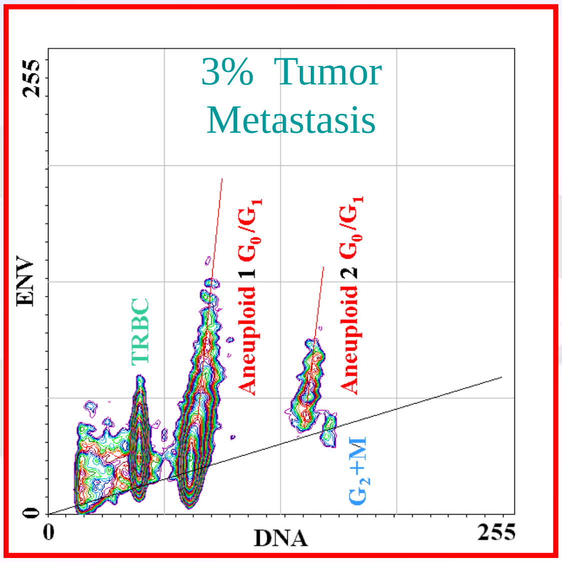
Krishan, et al. Cytometry 43, 2001

Nuclear Volume vs. DNA Content

Primary Breast Cancer



L.N. Metastasis



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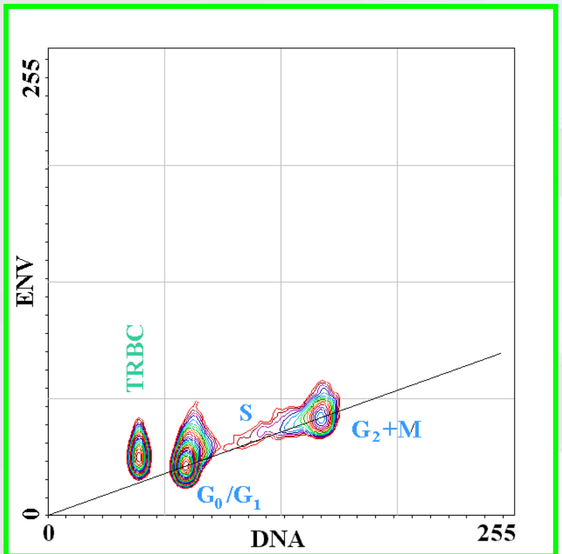
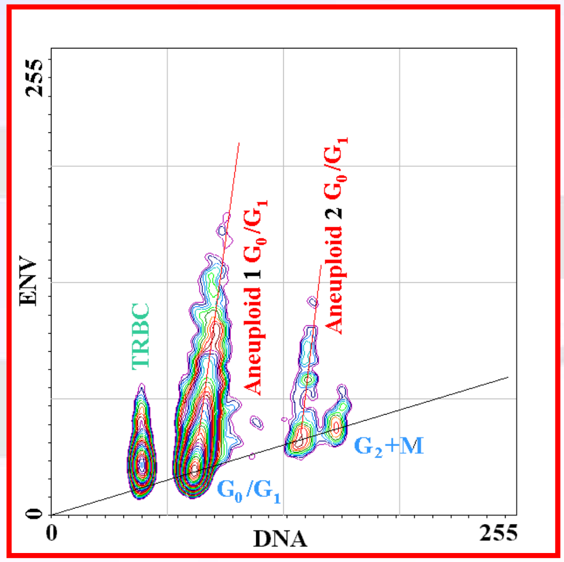
Krishan, et al. Cytometry 43, 2001

Nuclear Volume vs. DNA Content

Normal Colon



Primary Colon Cancer



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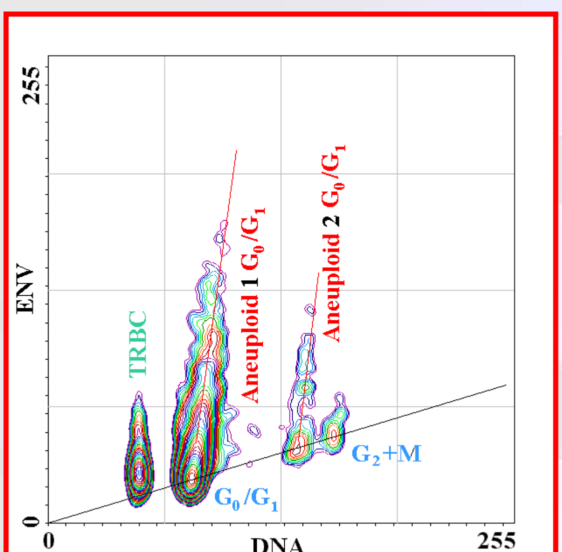
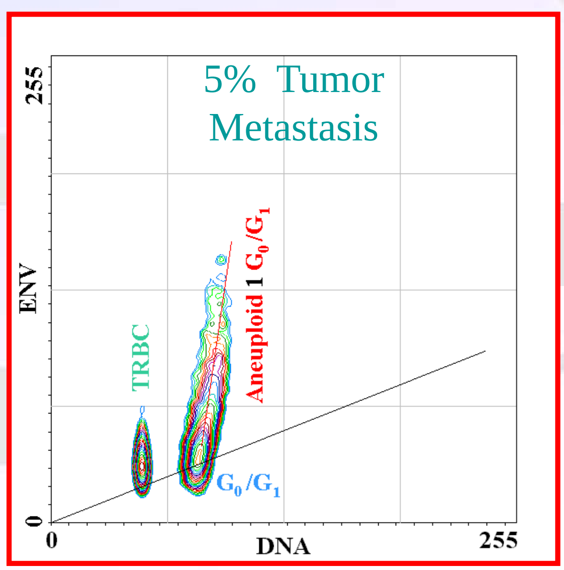
Krishan, et al. Cytometry 43, 2001

Nuclear Volume vs. DNA Content

Primary Colon Cancer



Colon Metastasis



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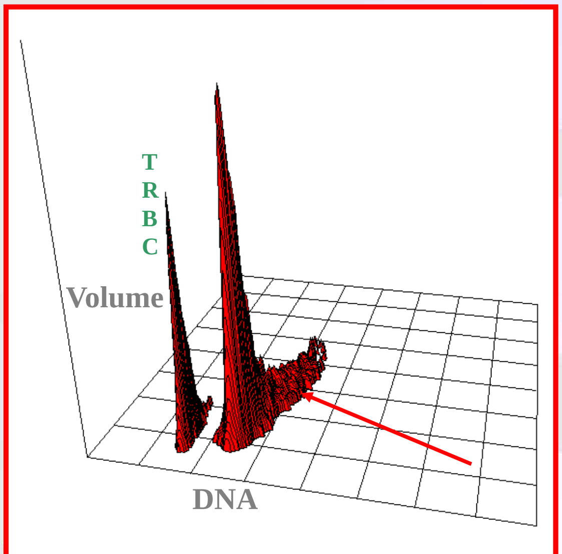
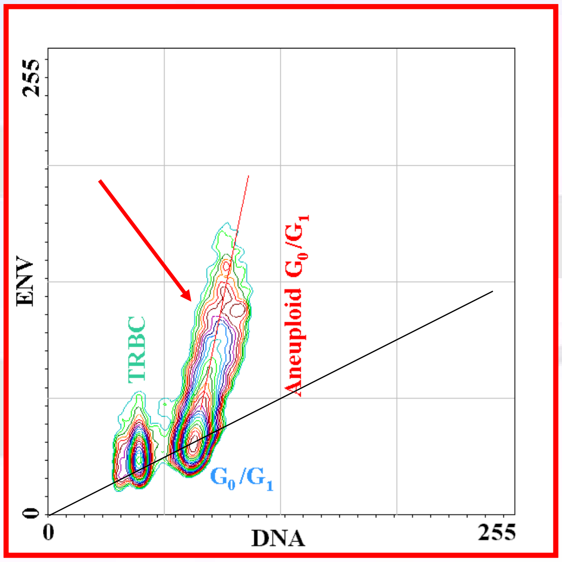
Krishan, et al. Cytometry 43, 2001

Nuclear Volume vs. DNA Content

Gastric Cancer





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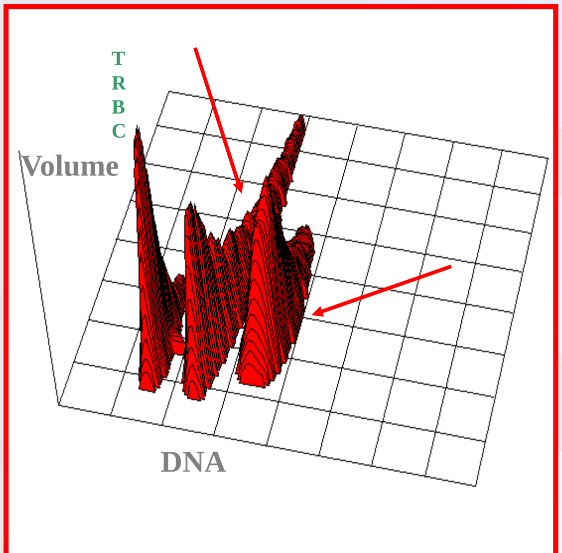
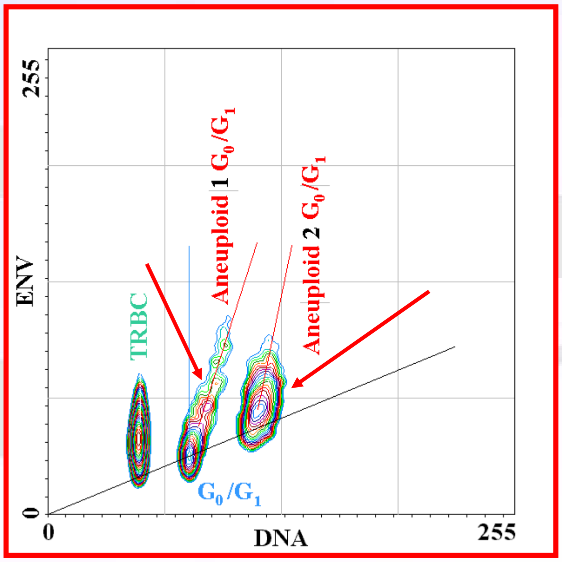
Nuclear Volume vs. DNA Content

Ovarian Cancer





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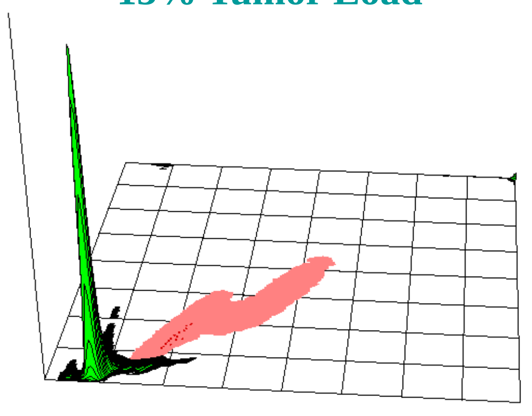
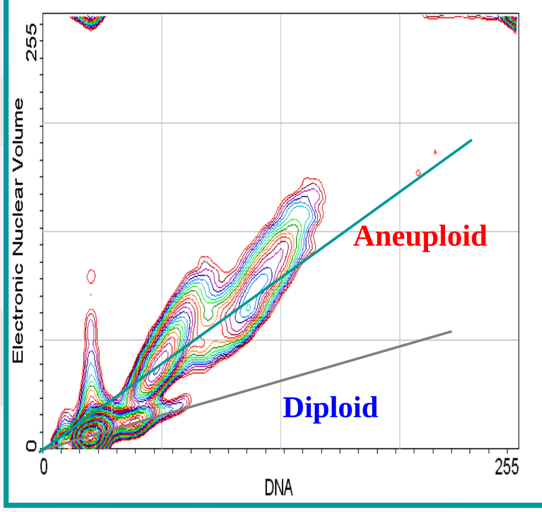
Nuclear Volume vs. DNA Content

Non-Small Cell Lung Carcinoma

13% Tumor Load





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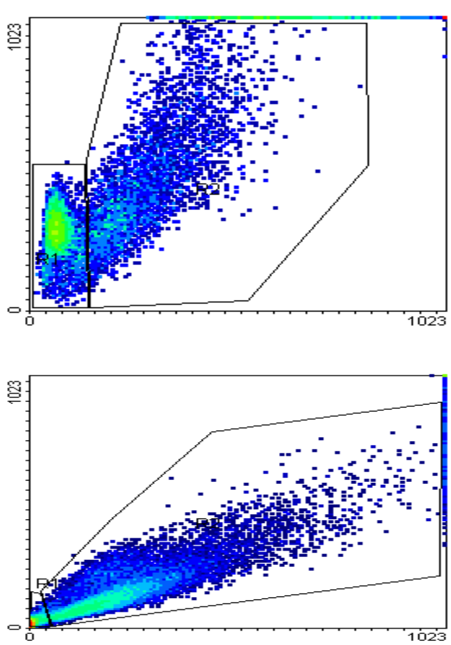
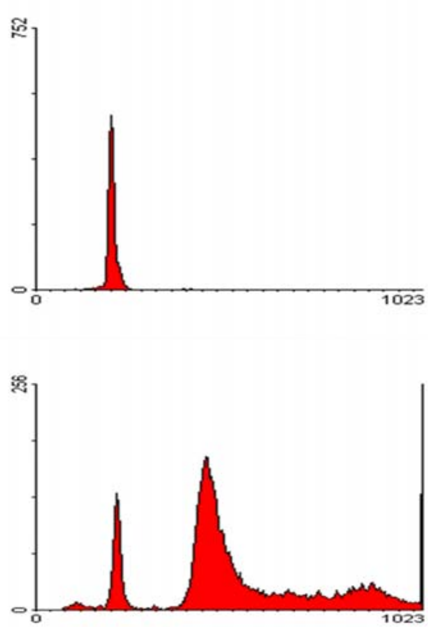
FORWARD SCATTER VS. DNA CONTENT

FORWARD SCATTER





SIDE SCATTER

DNA CONTENT

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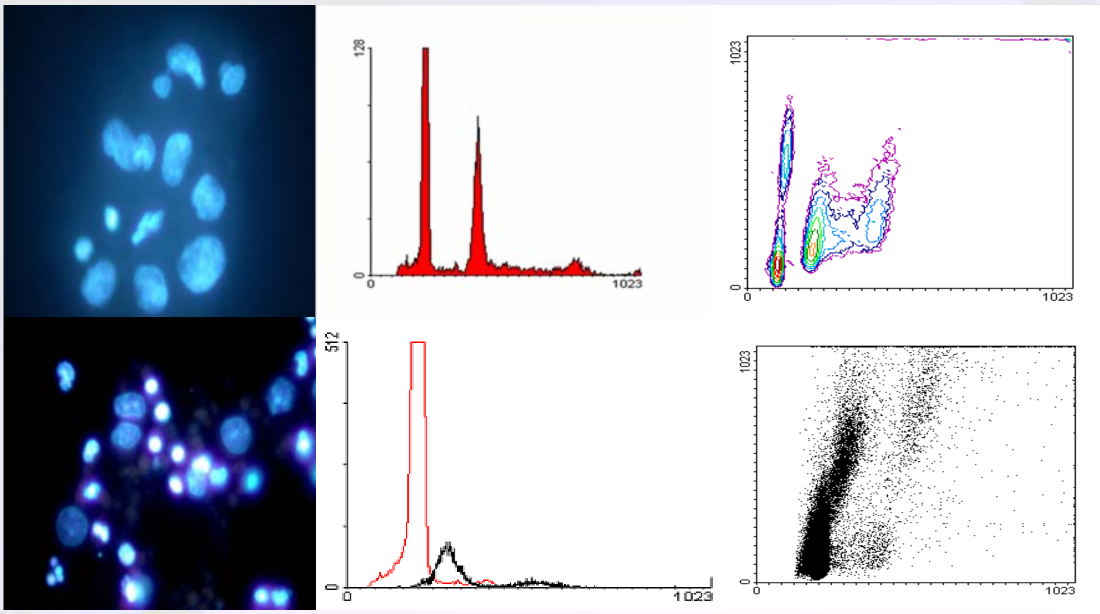
Aneuploid cells in a false negative peritoneal fluid

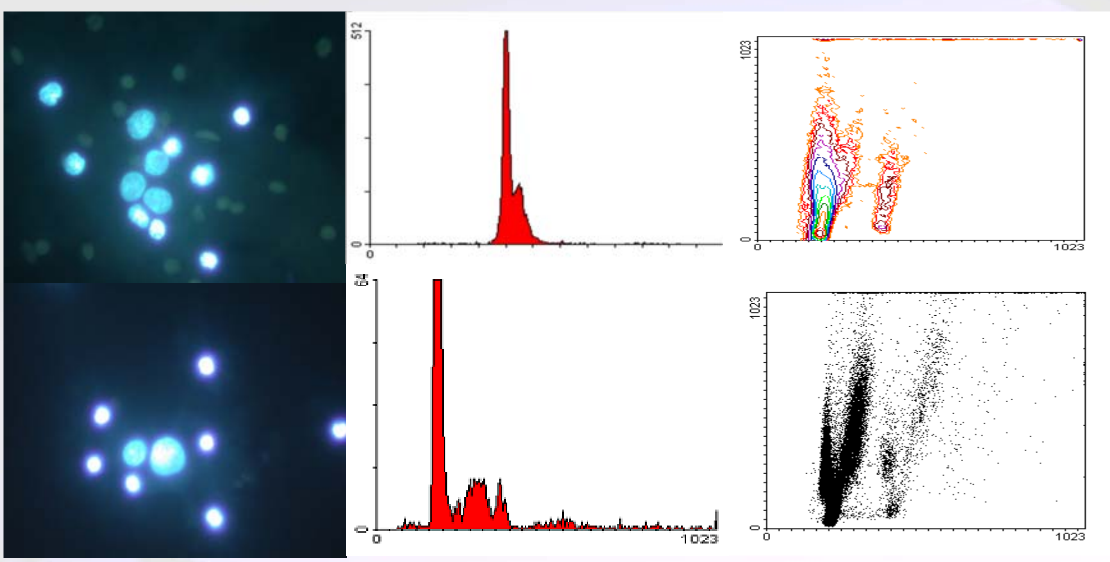


DNA Content

DNA vs. Protein

Krishan et al. Diag. Cytopath. 34; 528-541, 2006

Aneuploid cells in a false negative peritoneal fluid



DAPI

DNA Content

DNA vs. Protein

Krishan et al. Diag. Cytopath. 34; 528-541, 2006

DNA Flow Analysis of limited value?

- **Some studies reported that DNA flow cytometry was in general less sensitive than cytology for the detection of malignant cells and a higher percentage of false positives were seen by flow analysis.**

- **Based on these reports, it was generalized that DNA flow analysis did not offer any advantages over cytomorphology for the detection of malignant cells in body cavity fluids.**

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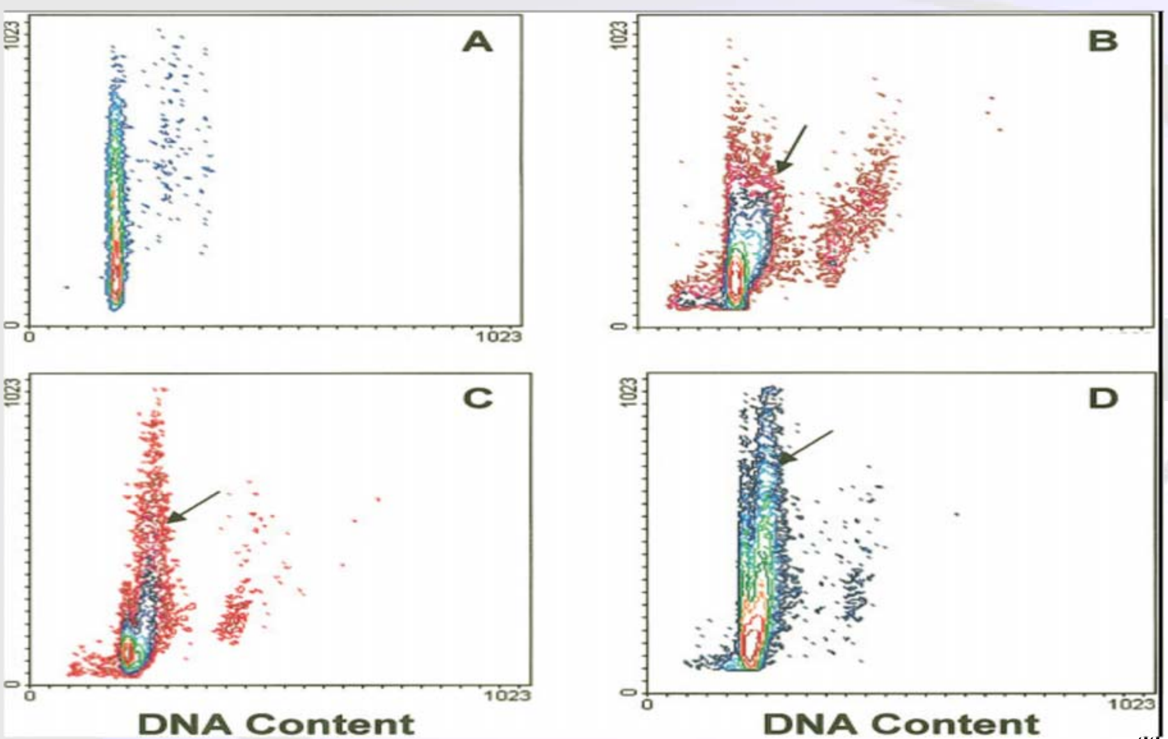
Hedley, et al., Eur.J. Clin. Onc. 20: 749, 1984

False Positive aneuploid cells in peritoneal fluid



False Positives in peritoneal fluid

- In some of the patients with liver cirrhosis or end stage liver disease, cells with large volume and "greater DNA fluorescence" are seen.
- These populations do not form a distinct peak in DNA histograms and may be caused by changes in chromatin density and fluorochrome binding rather than by the presence of true aneuploidy.

Krishan et al. Diagnostic Cytopathology,2006.

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DNA Cytometry and Immunocytology

- 130 body cavity fluids were examined for DNA aneuploidy and for expression of Epithelial Membrane Antigen by immunocytology (EMA-ICC).
- Sensitivity for detection of tumor cells was:
  - DNA aneuploidy alone = 38%
  - Cytology alone = 58.8%
  - Cytology and DNA aneuploidy = 73.5%
  - Cytology and EMA-ICC = 79.4%
- A combination of cytology and DNA aneuploidy had higher sensitivity than DNA aneuploidy alone (73% versus 38%).

Krishan, et al., Diagnostic Cytopathology, 34:528, 2006

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High Resolution DNA Cytometry, Conventional Diagnostic Cytology and EMA immunocytochemistry

- Out of 22 cytology positive samples, 20 were confirmed to be malignant on follow up.
- 4/8 suspicious samples with normal diploid DNA content had malignant cells.
- 7/15 samples with aneuploid cells were malignant and 8/15 were false positive.
- Most of the false positive aneuploid samples were ascites of patients with cirrhosis and liver disease.
- High resolution flow cytometry in combination with EMA immunocytochemistry reduced the false negatives from 41.2% to 14.7 %; an absolute reduction of 26.5% and relative reduction of 64.3%.

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Krishan A, et al. Diagn. Cytopathol. 34: 528-41, 2006.

Flow Cytometry in Diagnostic Cytology

Diagnostic Cytology

- Conventional Diagnostic cytology has a false negative @ 50%.
- Combination of diagnostic cytology with IHC can increase detection of tumor cells.
- Observer bias and small sample size can lead to artifacts.
- 24-36 hr are needed for results to be reported.

Flow Cytometry

- DNA Flow Cytometry has a false positive @ 58%.
- DNA Analysis in combination with IHC marker detection can increase sensitivity from 58 to 100%.
- Data is based on a large sample size and lack of observer bias.
- Data can be obtained in 2-4 hrs.

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Ber-EP4

- **Epithelial antigen.**
- **Expressed in:**
  - **75-90 % of carcinomas,**
  - **4% of mesotheliomas**
  - **0% of benign mesothelial proliferation.**

Comin CE, et el. Amer. J. Surg. Path. 31:1139-48, 2007.
Davidson B, et al., Diagn Cytopathol. 35: 568-78, 2007.

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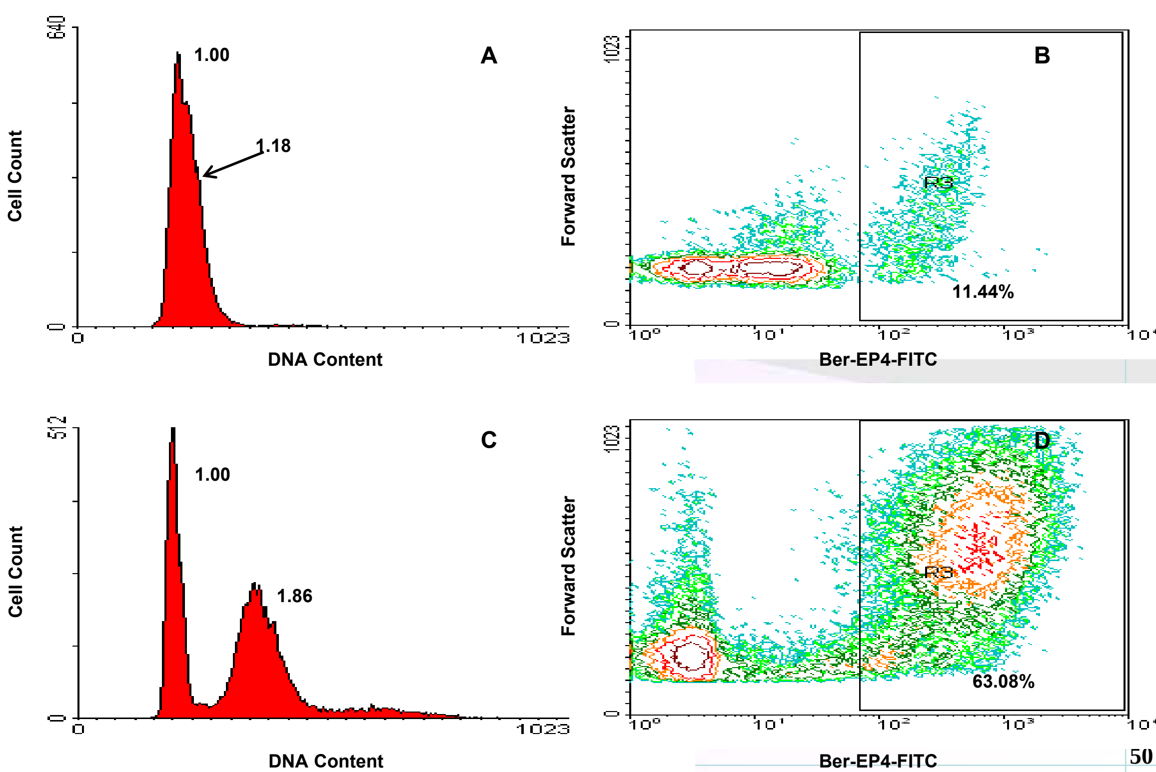
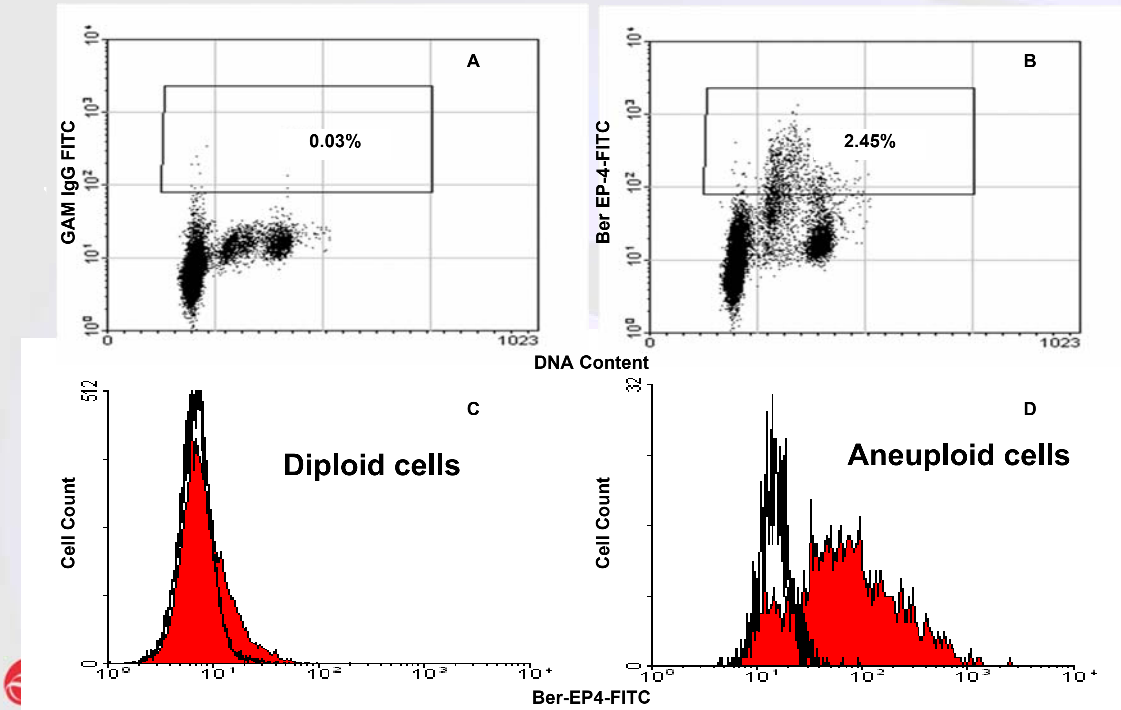
DNA Aneuploidy and Ber-EP4 Expression



Ber-Ep4 Expression in triploid cells from a peritoneal fluid



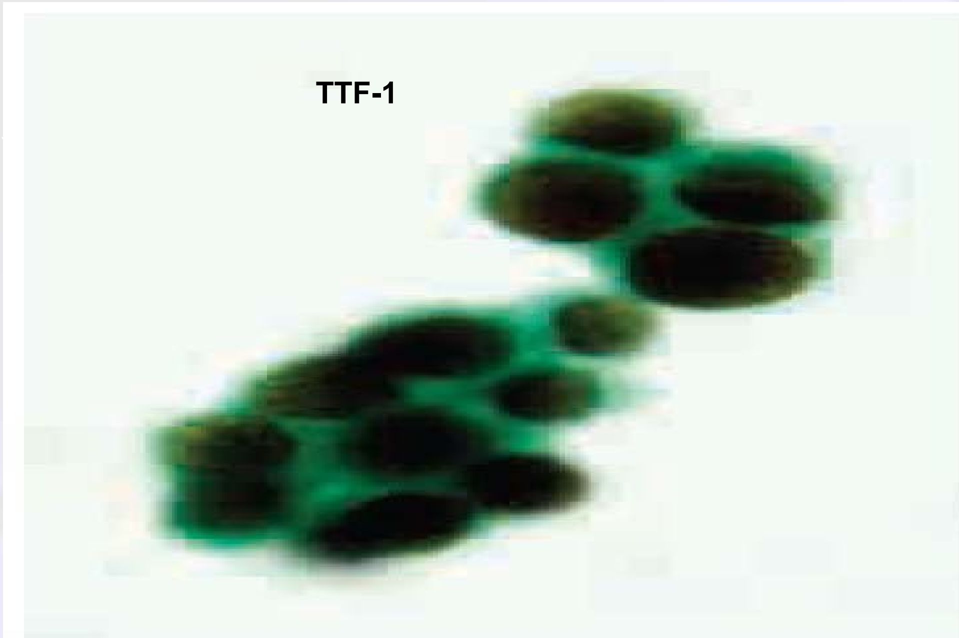
ISOTYPE vs. Ber-Ep4-Mob

Thyroid Transcription Factor-1

- A nuclear receptor found in 90% small-cell lung adenocarcinomas, ~23% of endometrial and endocervical ca. with negligible expression in squamous cell carcinomas.

- Siami, K., et al. Am J Surg Pathol. 31: 1759-63, 2007.
- Kalhor, N., et el. Mod. Path., 19: 117-23, 2006.
- Ordonez, NG, Mod Path. 19: 417-28, 2006.

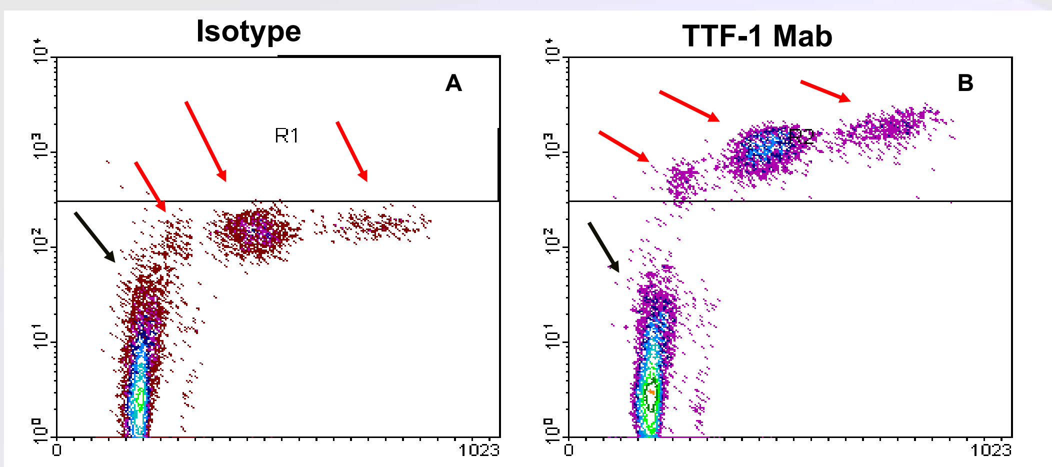
TTF1 positive nuclei in peritoneal fluid of a lung adenocarcinoma



Ganjei, Jorda & Krishan
Effusion Cytology, Demoss Medical

TTF-1 Expression in Aneuploid Cells from a Pleural Fluid

TTF EXPRESSION



DNA content vs. TTF expression in a human pleural fluid specimen stained with either the isotype or anti-TTF monoclonal antibody. Note the high expression of TTF-1 reactivity in cells with aneuploid DNA content.

Krishan et al. Diag. Cytopath. 34; 528-541, 2006

Flow Cytometric Monitoring of Marker Expression in Cells from Body Cavity Fluids

- **Fluids with aneuploid cells: 48/226 (21%)**
- **TTF-1 Expression: 45/150 (30%)**
- **Progesterone Expression ♀: 40/66 (60%)**
- **Ber-Ep4 Expression: 9/20 (45%)**

DNA Aneuploidy and Marker Expression

- Seventy-nine BCF were analyzed by flow cytometry for detection of aneuploidy and expression of Ber-EP4, progesterone, MUC4 or thyroid transcription factor-1.
- DNA index of equal to or greater than 1.2 was seen in 33/79 (41.7%) of the samples.
- By combining data on positive marker expression with that of DNA aneuploidy, the sensitivity for detection of malignant samples was increased from **58.5 to 100%.**

Krishan, et al., Cytometry Part A 77A: 132-143, 2010

Flow Immunocytology

- **Flow analysis can be used for rapid detection of the following diagnostic markers in cells from body cavity fluids:**
  - **Mucins (MUC1, MUC4, MUC16)**
  - **Epithelial Antigens (EMA, Ber-Ep4)**
  - **Calretinin** (mesotheliomas**)**
  - **Cytokeratins (CK7, 20)**
  - **Hormone receptors (ER, PR, VDR)**
  - **TTF-1** (adeno.ca of lung)
  - **P53, P63** (Squamous vs. adenoca of lung)
  - **Stem Cell Markers:** CD34, CD90, CD117, CD133, CXCR4 ALDH1, CD44+/CD24- phenotype

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Flow Cytometric Analysis of Cells in Body Cavity Fluids

Conclusions

- **High resolution flow cytometry can be used for rapid identification of cells with aneuploid DNA content.**
- **Nuclear volume and protein content can be used to differentiate between normal and tumor cells with diploid DNA content.**
- **Specific marker expression (e.g., ER, EMA, TTF-1) can be used to suggest a possible site of origin.**
- **Multiparametric flow analysis may be able to reduce the false negatives in body cavity fluid cytology.**

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Tumor Stem Cell Marker Expression in Cells from Body Cavity Fluids

Ber-EP4
TTF-1
ALDH-1
CD44+
CD24-

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Tumor Stem Cell Markers

- **ALDH1 is expressed in both hematopoietic and tumor stem cells.**
- **CD44+/CD24-/CD133+ phenotype is characteristic of breast cancer stem cells.**

Wright, MH et al. Breast Can. Res. 10: R10, 2008
Ginestier, C et al., Cell Stem Cell. 1: 555-567, 2007
Sheridan, C., et al., Breast Can. Res. 8: R59, 2006

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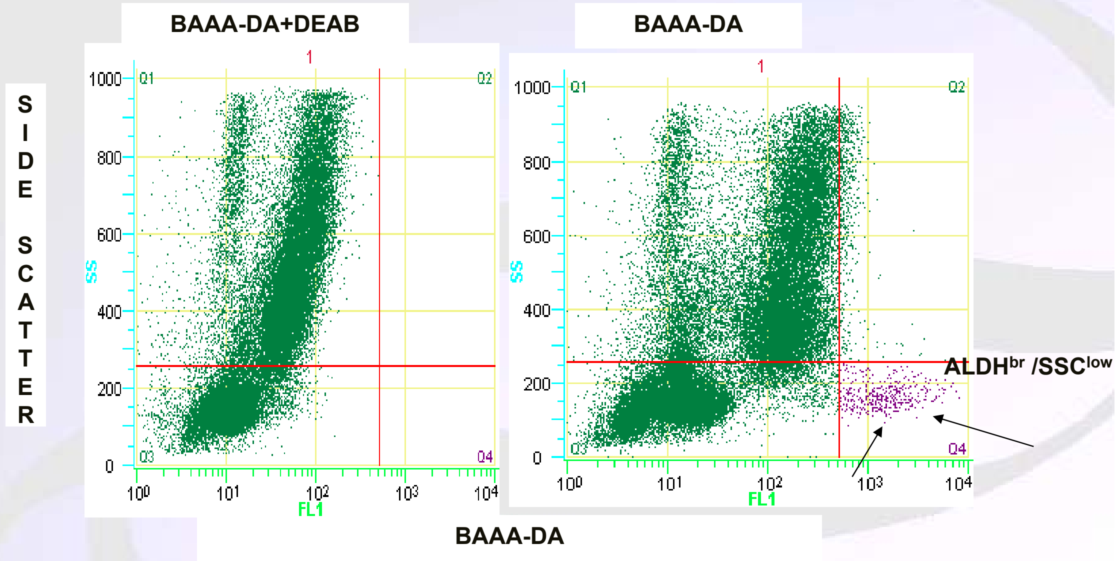
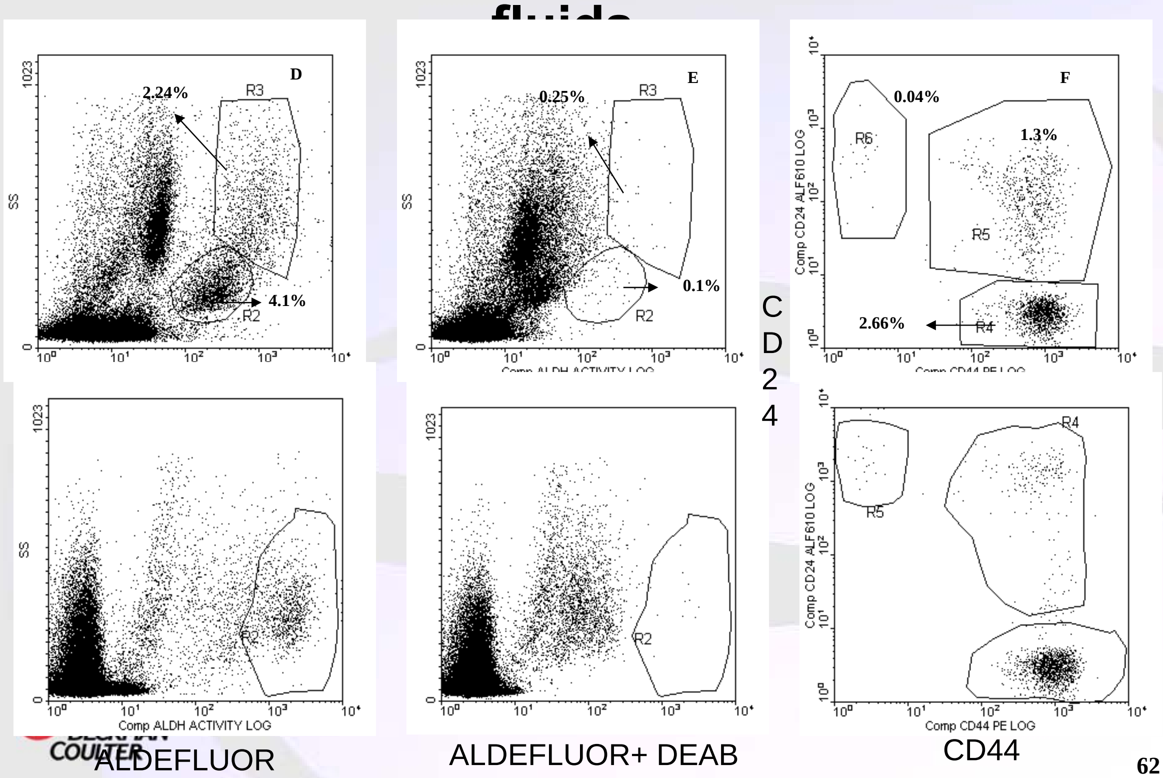
ALDH+ Over-expression in Cells with Small side scatter



BAAA-DA = BodipyTM-aminoacetaldehyde diethyl acetal {ALDEFLUOR}
DEAB = Diethylamino-benzaldehyde

ALDH1, CD44 and CD24 Expression in cells from body cavity fluids

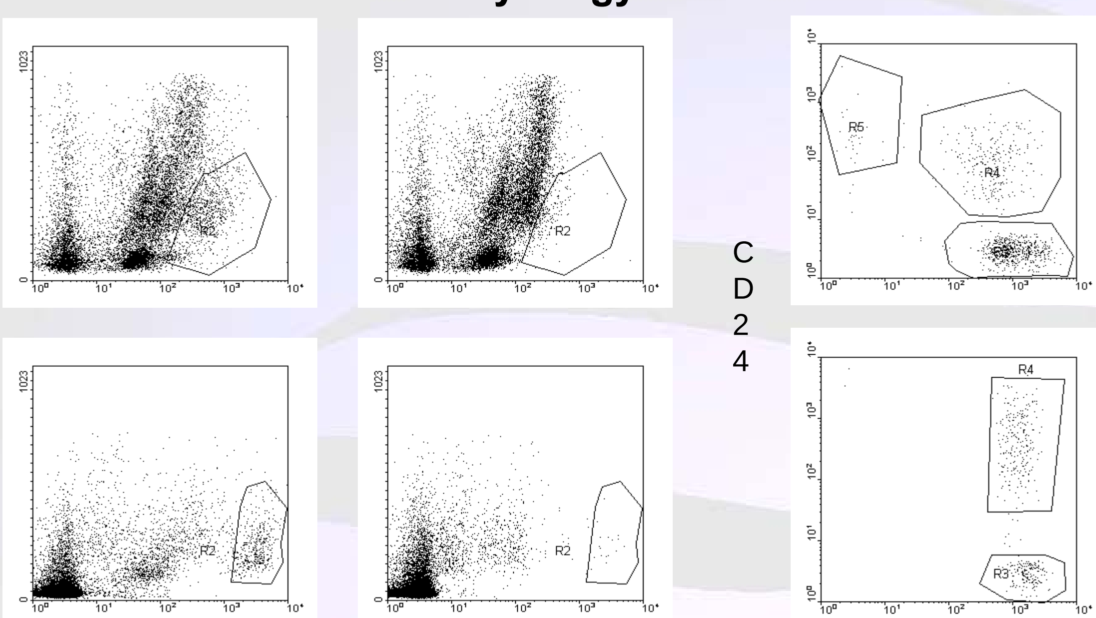


Tumor Stem Cell Analysis in Body Cavity Fluids

- **CD34/CD45 cells**
- **CD90, CD117, CD133**
- **CXCR4 Expression**
- **Electronic Cell Volume of Stem Cells**
- **Side Population (SP cells)**
  - **Hoechst 33342**
  - **Vybrant DyeCycle Violet V35003**
- **ALDH1 positive Cells**
- **CD44+/CD24-**

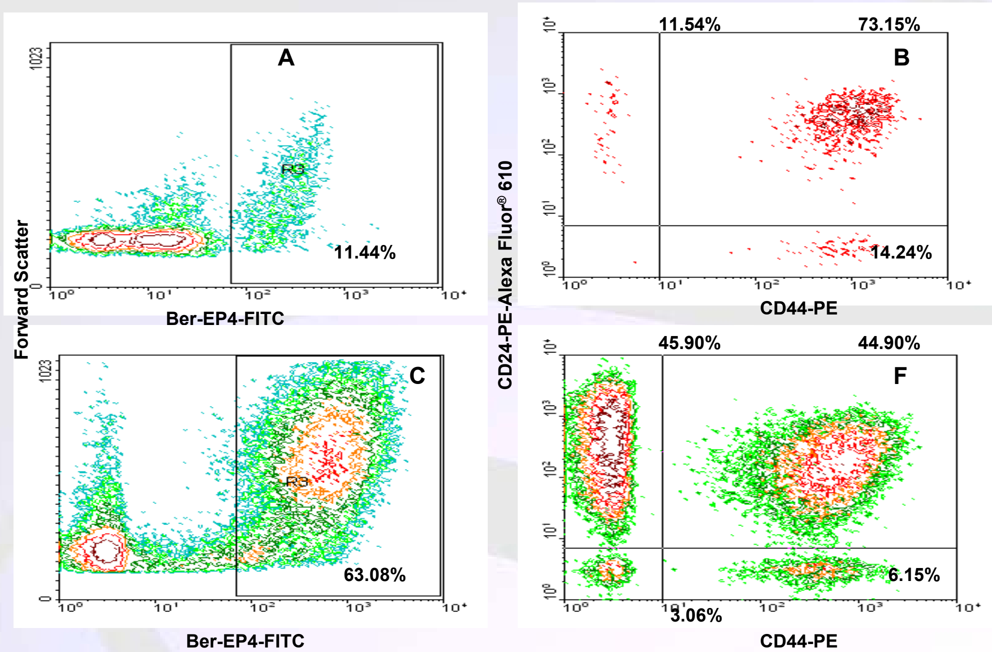
Cytometry A 73: 160-67, 2008
Cytometry B: April, 2008

ALDH1, CD44 and CD24 Expression in body cavity fluids with negative cytology



ALDEFLUOR ALDEFLUOR+ DEAB CD44

CD44+ and CD24- Expression in Ber-EP4+, cells from a benign and a malignant body cavity fluid.



BCF 112, 123 fig 1 BCF2

ALDH1, CD44 and CD24 Expression

- **In peritoneal and pleural fluids of patients containing malignant cells, ALDH^{bright} cells with SSC^{low} and SSC^{high} and CD44^{+}/CD24^{-} expression are seen.**
- **However, similar cells were also seen in body cavity fluids of some of the patients who had negative cytology and did not have a proven malignancy.**
- **The presence of ALDH^{bright} cells with CD44^{+}/CD24^{-} expression in inflammatory and benign body fluids needs further evaluation.**

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