Computerized tools in image cytometry- slide based cytometry or Virtual Flow Cytometer for Cancer Diagnostics

Hernani Cualing MD

Medical Director IHCFLOW, Inc

Cell based markers and Personalized profiling- Pathology Cancer Reports as Quantitative Profile of Prognostic Markers

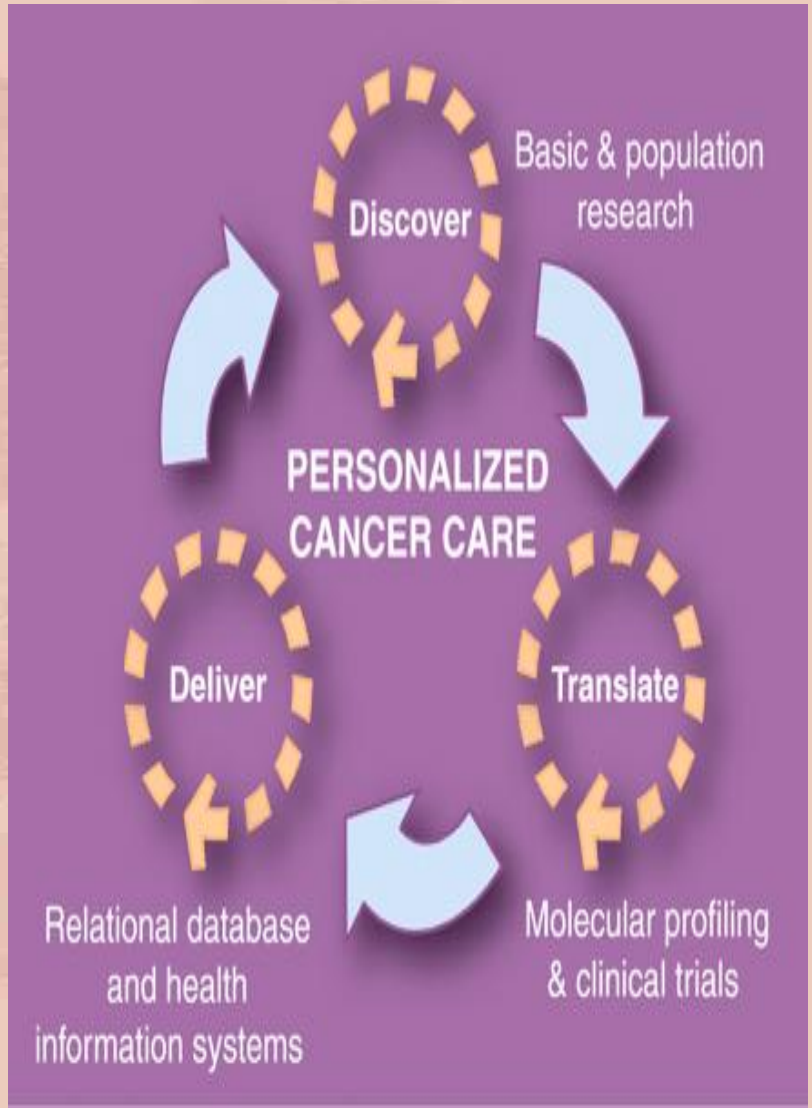
The emerging use of cancer biomarkers may herald an era in which physicians no longer make treatment choices that are based on population-based statistics but rather on the specific characteristics of individual patients and their tumor.

Cancer Biomarkers—An Invitation to the Table

William S. Dalton[1]* and Stephen H. Friend[2]*

Science 26May 2006: Vol. 312. no. 5777, pp. 1165 - 1168

CEO- MOFFITT Cancer Center



Your Current Perspective

Cancer incidence rates

10 million new cases each year. Expected to rise to 15 million by 2020

Almost half are from the US, Europe and Japan

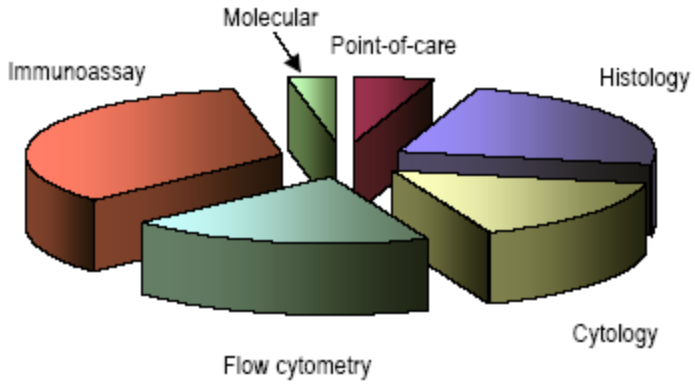
Cancer incidence is growing rapidly in the developing world

Histological analyses of biopsies continues to be the mainstay of cancer diagnosis

Advanced staining techniques such as IHC and ISH

- Laboratory automation
- Expansion of target bio-markers
- Targeted cancer therapuetics

Worldwide Market for IVD Cancer Diagnostics



Sample Preparation

Grossing

+

Processing

+

Embedding

+

Sectioning

→

Tissue Staining

Routine, H&E/SS

(+)

Advanced, IHC/ISH

→

Analysis

Pathologist

(+)

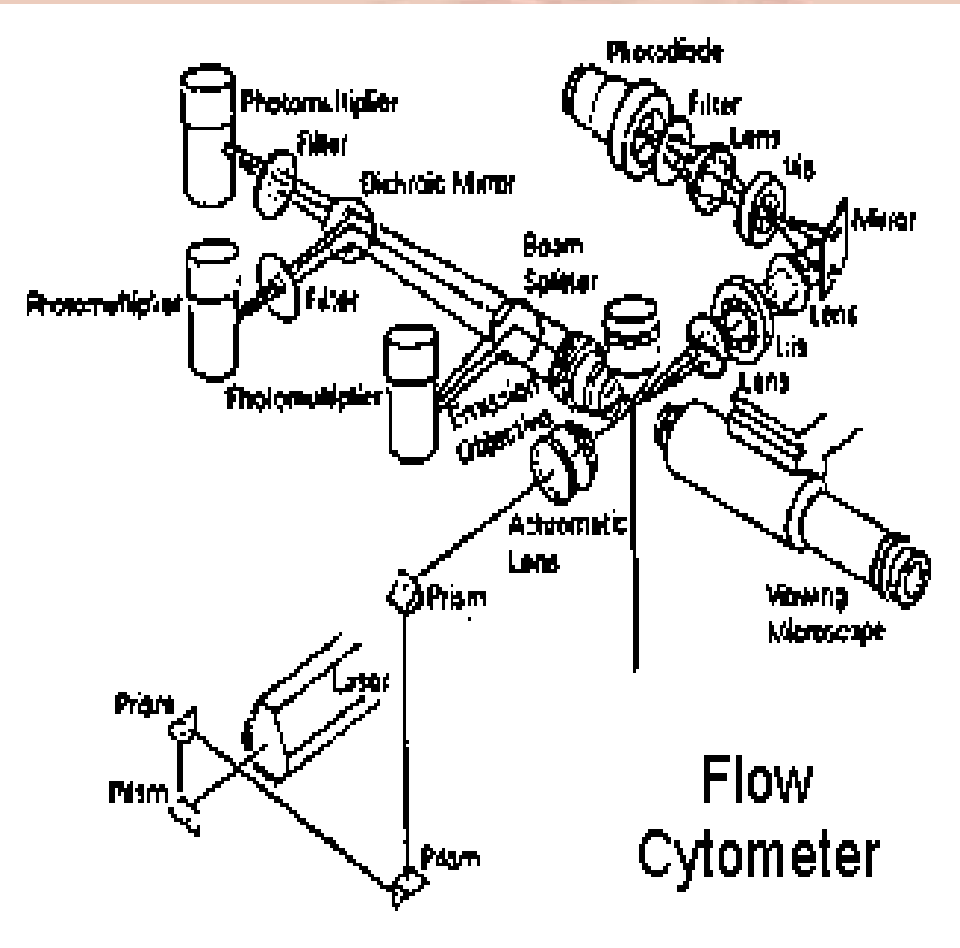
Image Analysis

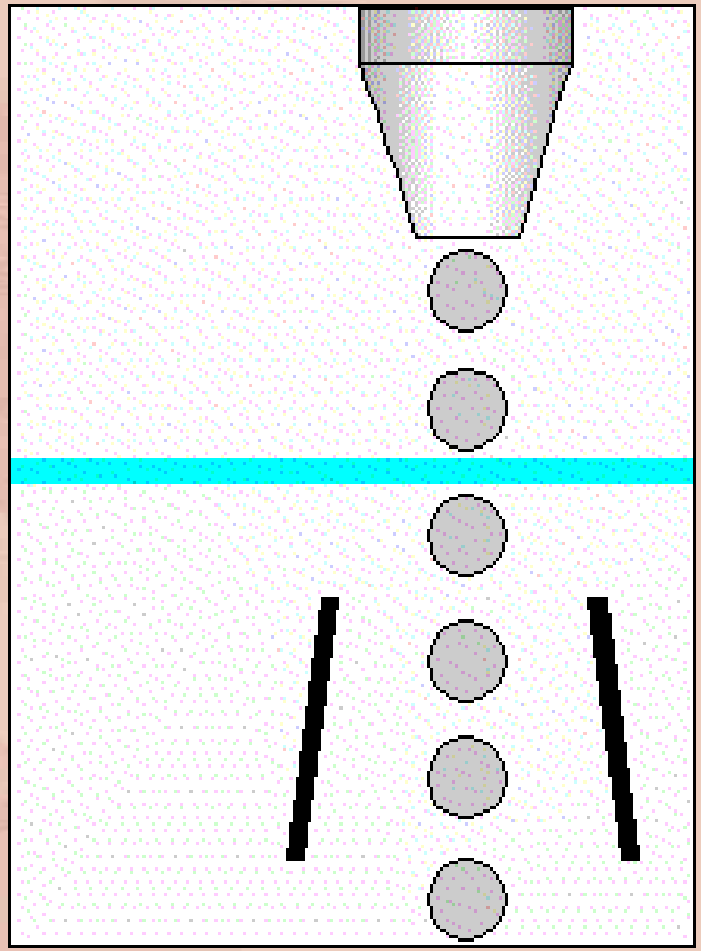
My Perspective:
Slide based cytometry promises to be a tool to complement flow cytometry and enhance objectivity in histologic analysis

Niche:
1.For tissue and cell-based quantitative analysis of immunohistochemistry stained cells for a marker profile

2. Logical extension of automation in histology and immunohistochemistry- need a tool to convert ihc to objective result reporting

INTRO: Flow cytometry Clinical Standard of Marker Profiling

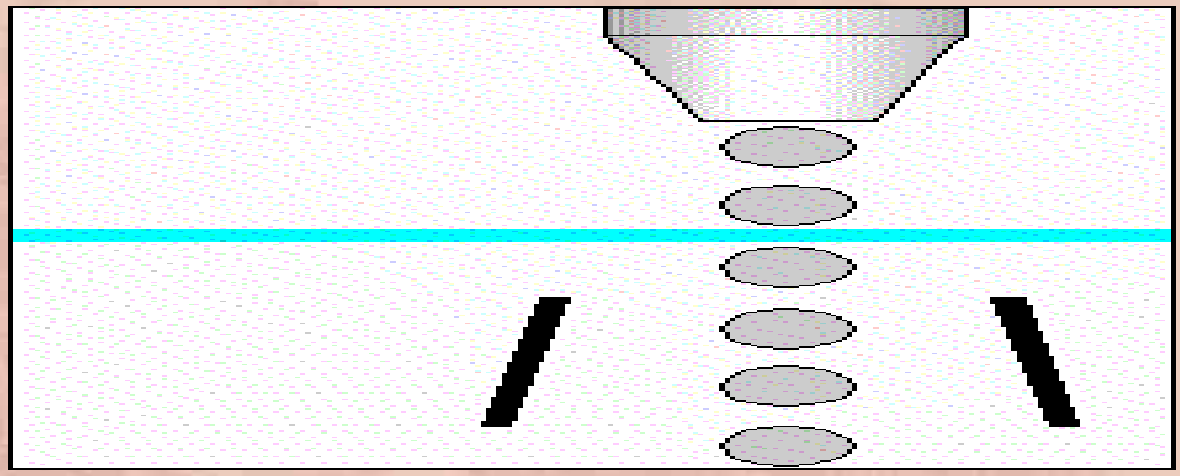




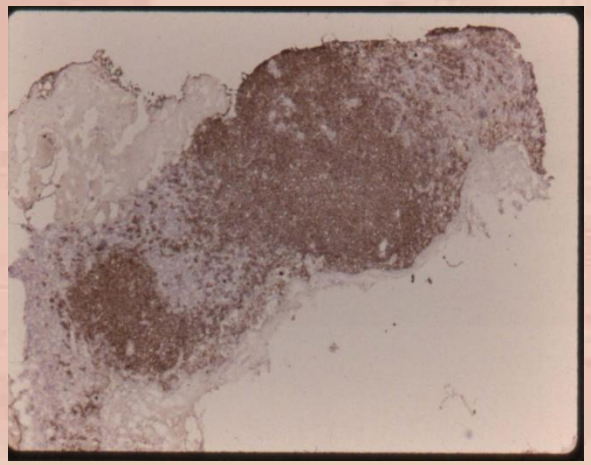
Clonality of tumors

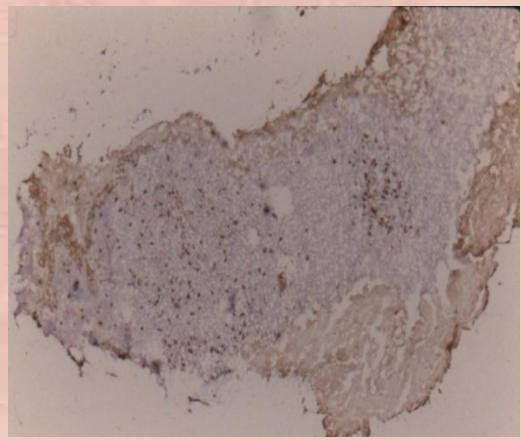
MARKERS

- **IMMUNOPHENOTYPE**
  - **flow cytometry**



- **immuno-**
- **histochemistry-”stains for chains”**





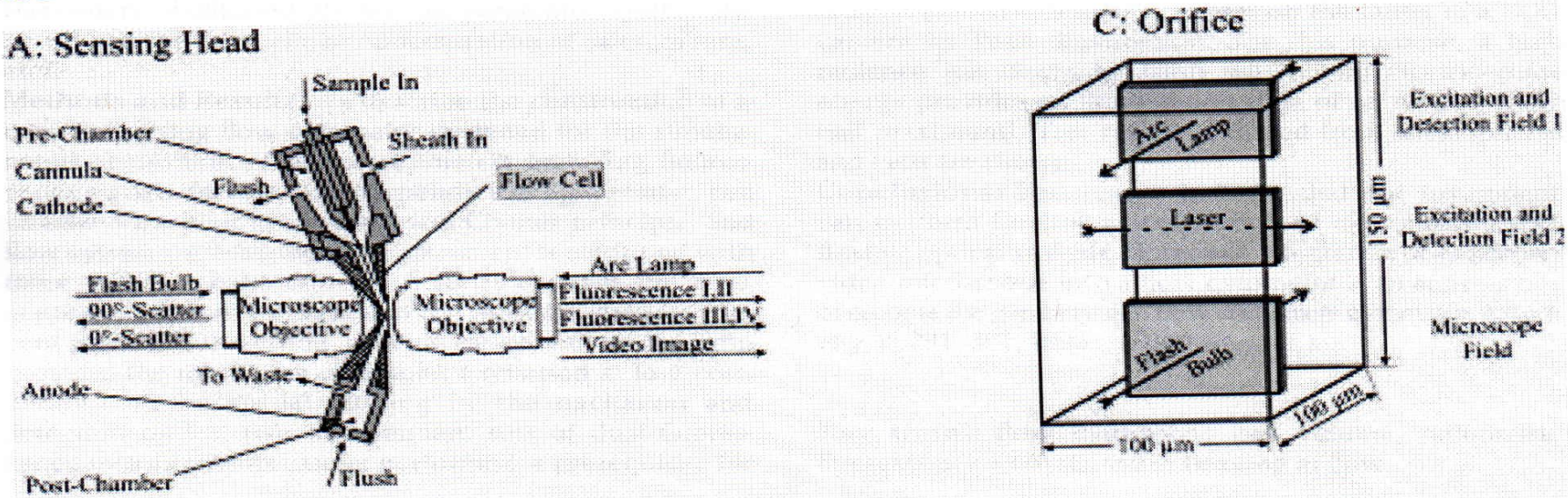
A New Multiparameter Flow Cytometer: Optical and Electrical Cell Analysis in Combination With Video Microscopy in Flow

Joachim Wietzorrek,[1,2]* Nikolaus Plesnila,[2] Alexander Baethmann,[2] and Volker Kachel[1]

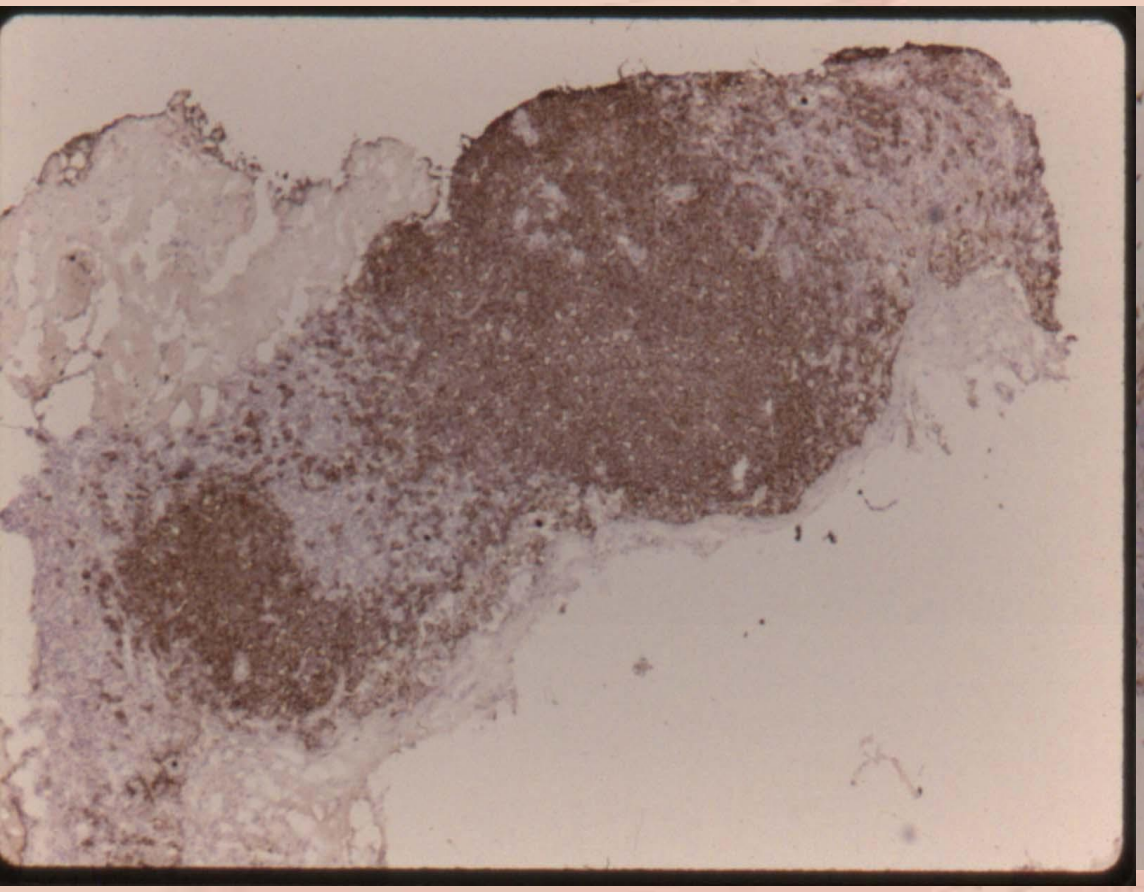
[1]Max-Planck-Institut für Biochemie, Martinsried, Germany

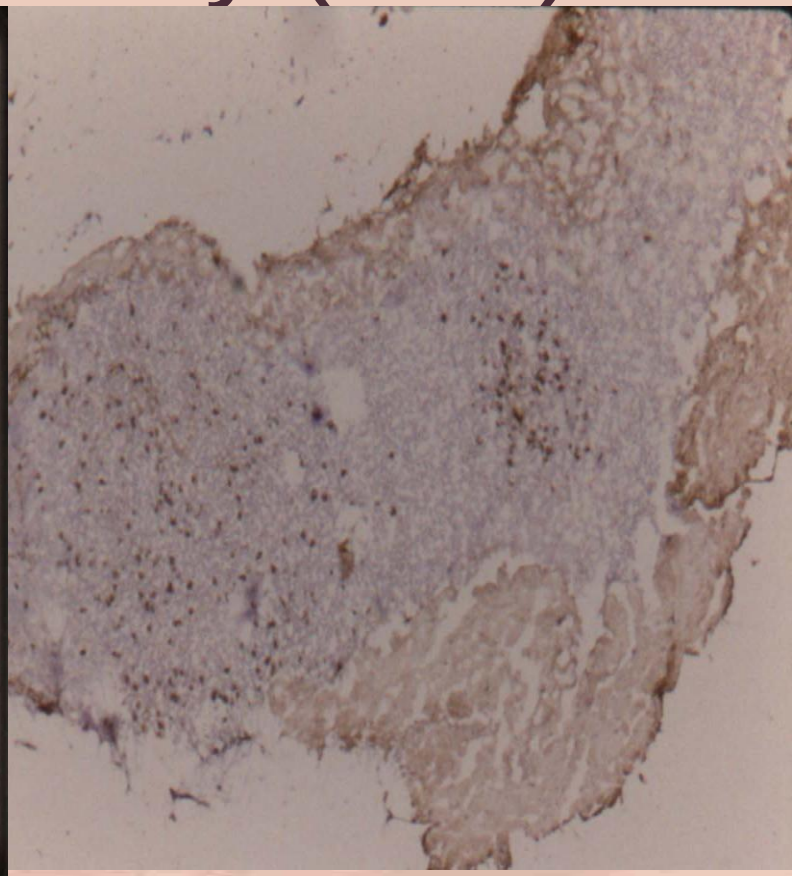
[2]Institut für Chirurgische Forschung, Klinikum Großhadern, Ludwig-Maximilians University, Munich, Germany

Received 13 July 1998; Revision Received 27 October 1998; Accepted 15 November 1998



Immunohistochemistry (IHC)



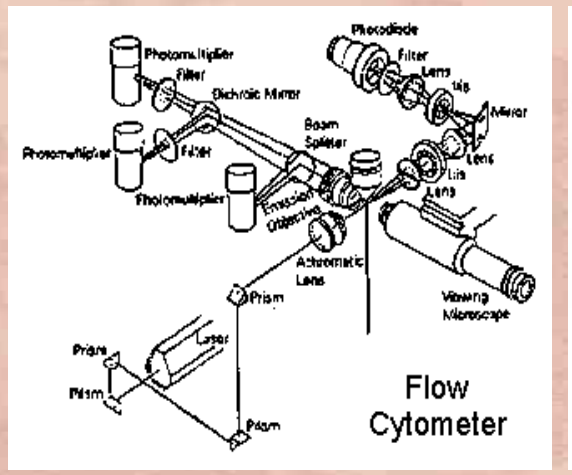


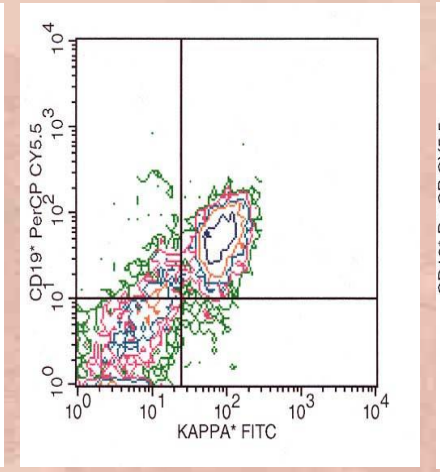
How can we obtain a set of positively-stained cells and a set of unstained “same-type” cells in tissue analysis.

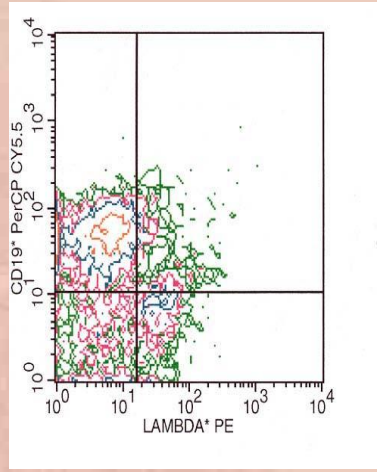
Flow cytometry and immunohistochemistry-parallels

MARKERS

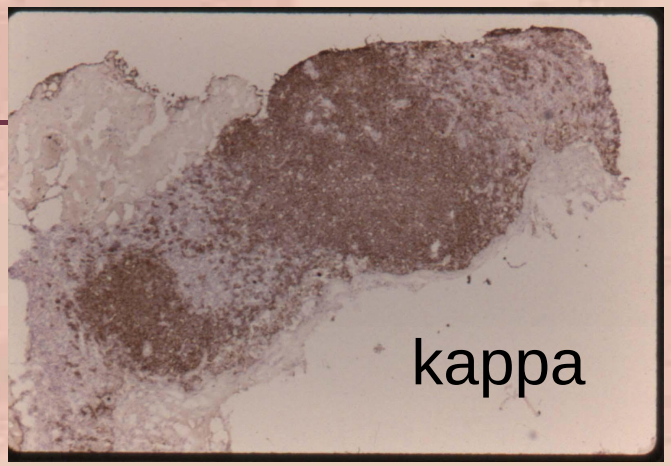
- IMMUNOPHENOTYPE
  - flow cytometry

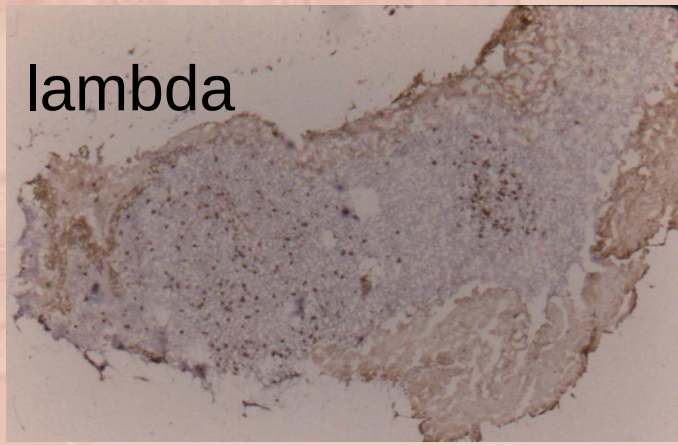






- Immunohisto-chemistry





Billable codes

- “Beginning Jan 1, 2005, CMS immunohistochemistry code 88342 is replaced by 88360 for manual or semiquantitative grading and…. code **88361** for computer assisted technology (digital cellular imaging- includes computer **software** analysis of stained microscopic slides” AMA bulletin
- *Quantitative immunohistochemistry- Morphometric analysis, tumor immunohistochemistry (quantitative or semiquantitative, each antibody; using computer-assisted technology- 88361.*
- *In situ hybridization computer assisted quantitative-**88367**, manual is 88368*
- Current practice-estimate % results and report results- bundled with report bill

Applications

- **Prognostic markers**-Counting tumor infiltrating lymphocytes (CD8 TILs in B-cell lymphomas and Carcinomas- importance of host response genes highlighted by gene microarrays
  - ER/PR Her2Neu score– Cell Analysis System Inc, Cell Quant.
- **Diagnostic- IHC marker antigens- routine Diagnosis**
  - Counting antigen loss in T cell lymphomas,ie, MF CD7
  - Kappa Lambda ratio in B cell lymphomas
  - Proliferative Index (Ki67) as measure of aggressiveness
  - CD34/CD117 estimate in MDS/Leukemia bone marrow trephine core biopsy

Therapeautic- Targeted antigens in Clinical Trial

  - Monitoring CD2 or CD30 pre- and post-therapy AntiCD2,CD30- Medimmune Anti CD2 Medarex AntiCD30
  - Big Ticket- Monitoring CD20 post Rituxan therapy

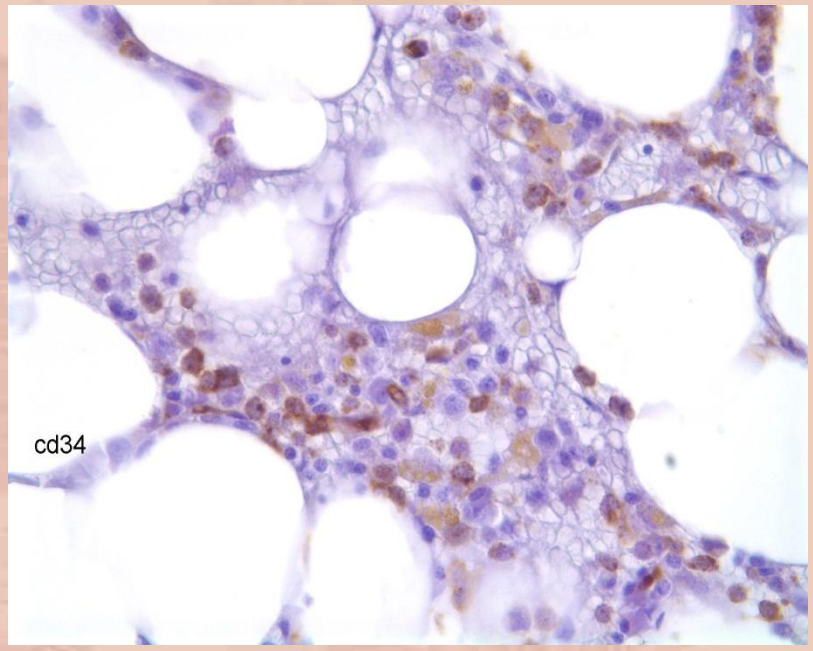
Quantitative BM IHC Report:All BM biopsy iHC are estimates

IHC ANALYSIS

Antibody/Test	Marker For	Results
CD34/QBEND-10	Endothelium, Stem Cells, Blasts, GI Stromal Tumor	Few Cells Positive, <5%
CD117	Myeloid and Mast Cells, Gastrointestinal Stromal Tumor (c-kit)	Few Cells Positive, ~5%
MPO	Myeloperoxidase, Myeloid Lineage Cells	Myeloid Cells Positive
GPH-A	Glycophorin A, Erythroid Cells (JC 159)	Erythroid Cells Positive
MURA	Muramidase, Lysozyme	Myelo/monocytic Cells Po
CD68	Macrophages and Myeloid Cells (KP1)	Histiocytes Positive
CD61	Glycoprotein IIIa, Megakaryocytes, Platelets	Megakaryocytes Positive

INTERPRETATION:
MARKED MYELOID HYPERPLASIA WITH SLIGHT INCREASE OF CD34+ BLASTS (<5%).

Reported by: CDW/ca



Above example is the current semiquantitative reports based on estimate- we are used to the the subjective, estimation that we disregard this mode as real potential for error propagation in diagnosis and prognosis

General Issues in the problem domain of immunohistochemical interpretation and analysis

1.Current quantitative IHC answers the question of antigen density and not population statistic.

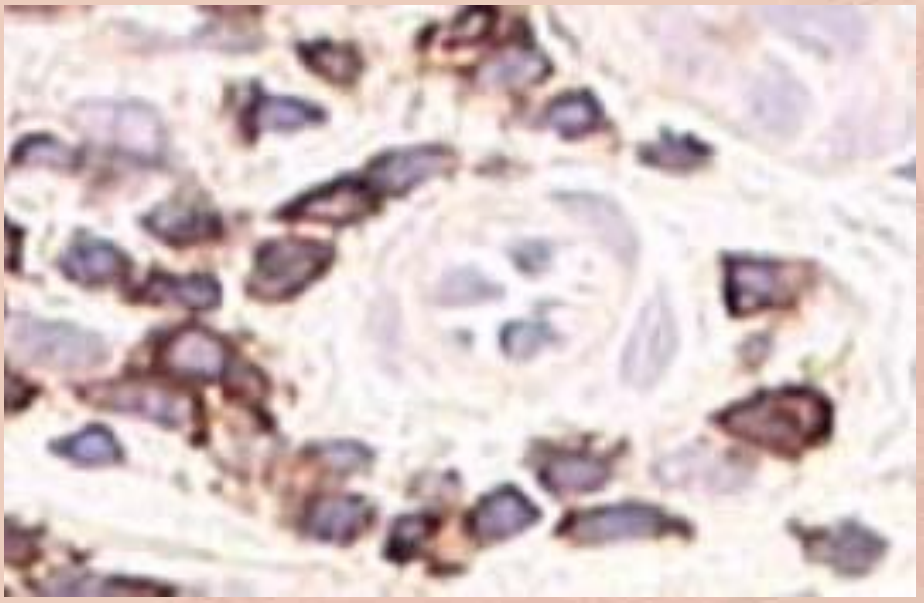
2. Lack of method standardization and objective interpretation as well as method of integration into diagnostic process

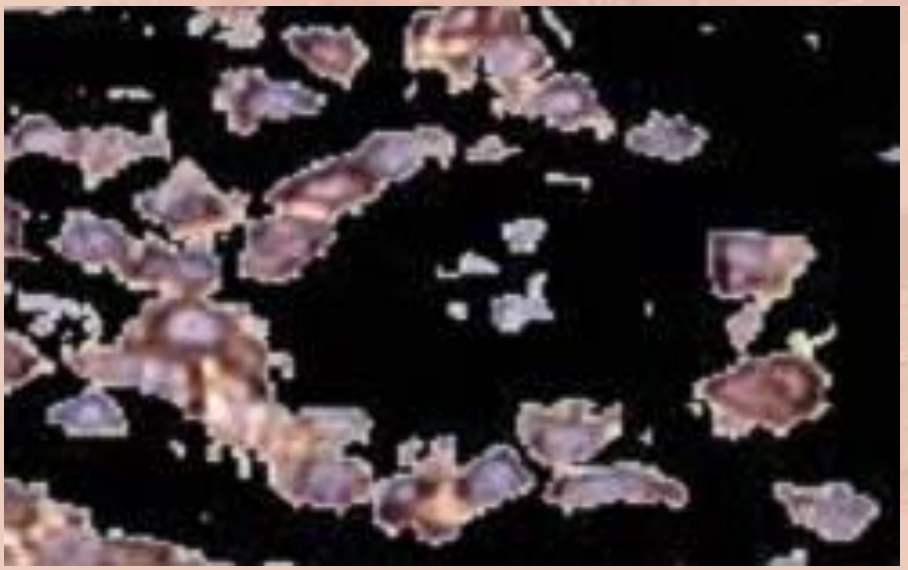
3. Current practice of guessing the percent positive

4. Not all submitted biopsy for interpretation are suitable for flow cytometry

5. No tool available for pathologist to convert immunohistochemistry to flow cytometry-like results

Automated detection of chromogen stained biological cells in tissue in a population statistic manner has lagged behind quantitation of antigen in tissue and cells for prognosis and diagnosis

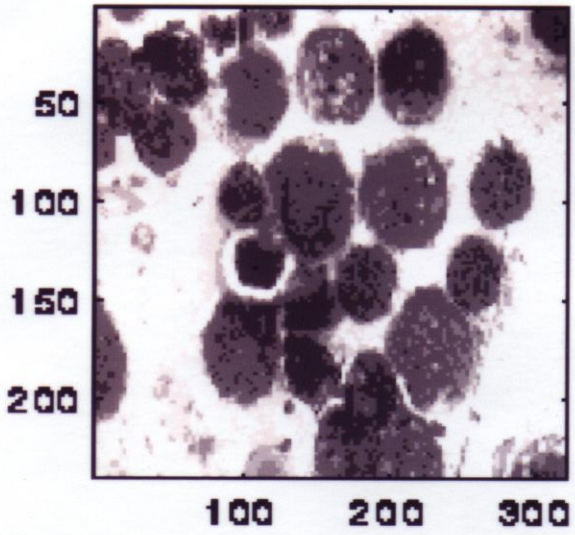


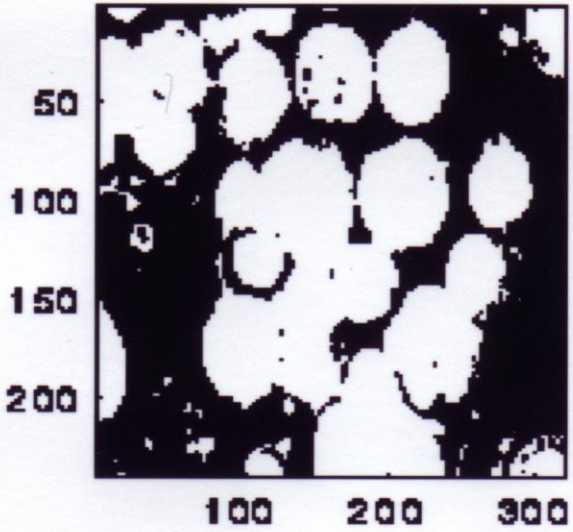


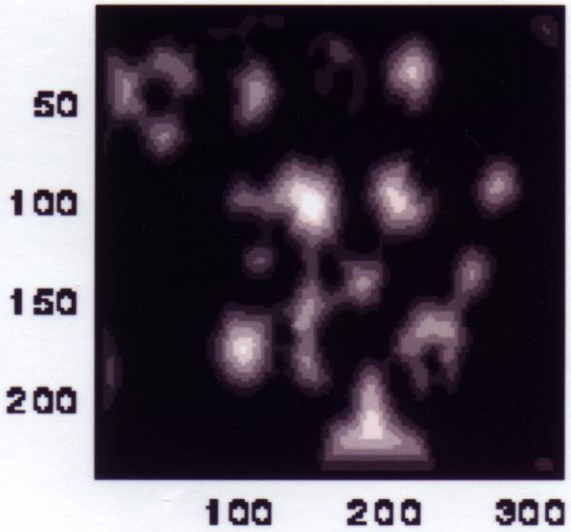
Four Principles of Image Analysis.

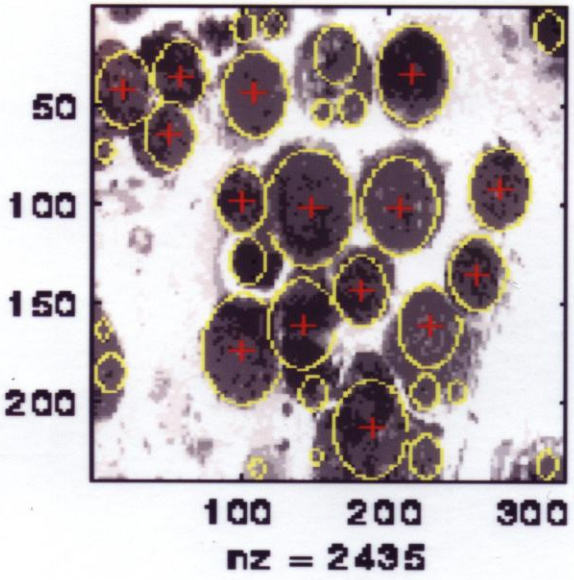
- Feature invariance or *invariance*
- Dimensionality reduction- *reduction of information*
- *Absence of distortion,*
- *and relationship to verbal descriptors.*

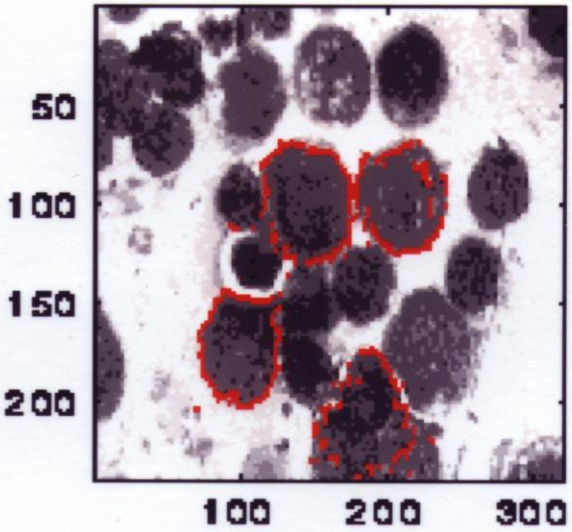
Image analysis (grayscale) on benign and cancer lymphocytes











- Thresholding
- Watershed
- Identifies centroids
- Grows region to edges
- Segments cells
- Separate overlapping cells
- **FEATURE EXTRACTION**
- **CLASSIFIER**

10 > 2-dimensions 65 > 2 dimensions

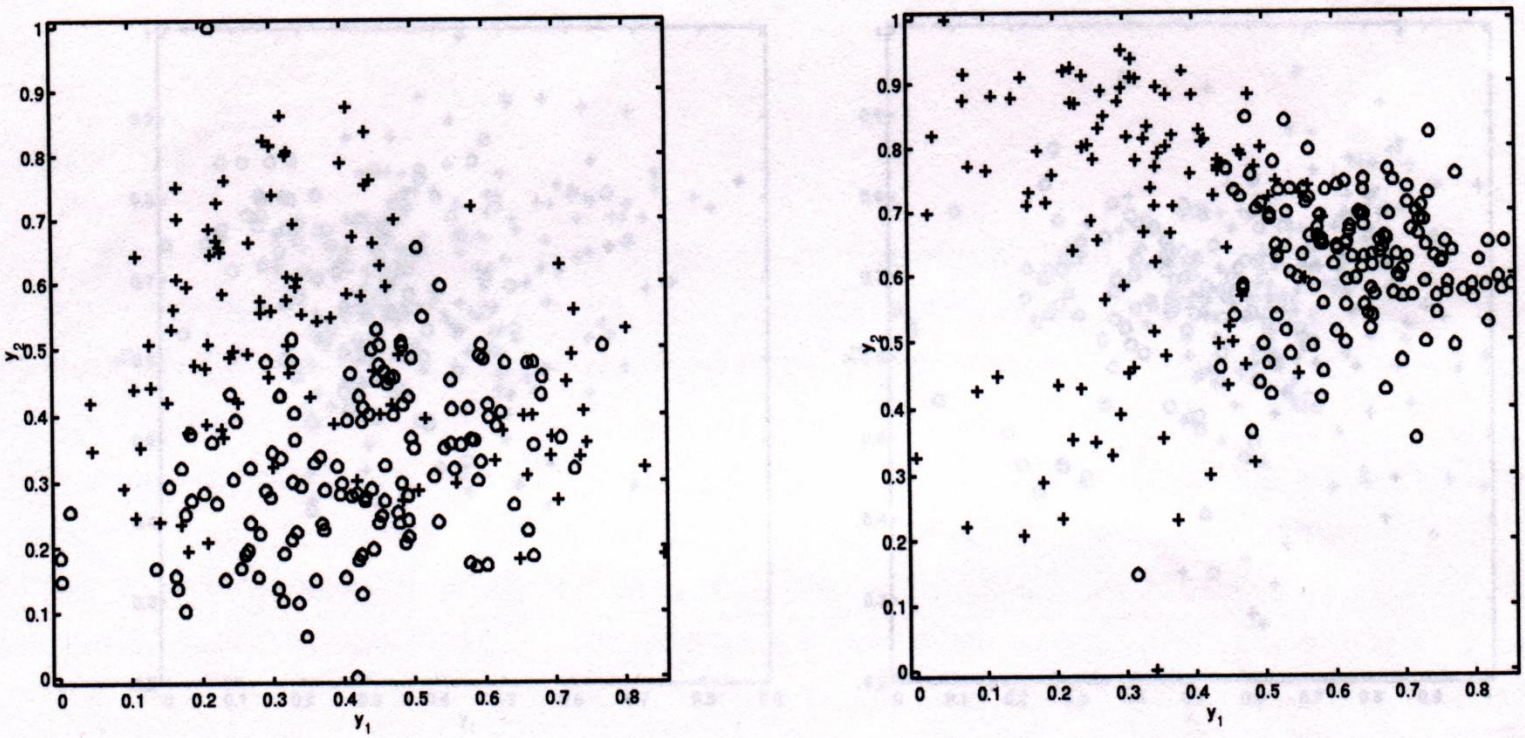


Figure 5: Separability of benign cytology (represented by a ∘) from low grade lymphoma (represented by a +). The left panel shows the separability in 2 dimensions from the original features, while the right panel shows the separability in 2 dimensions of the data comprising the original features and distinct quadratic combinations of the original features

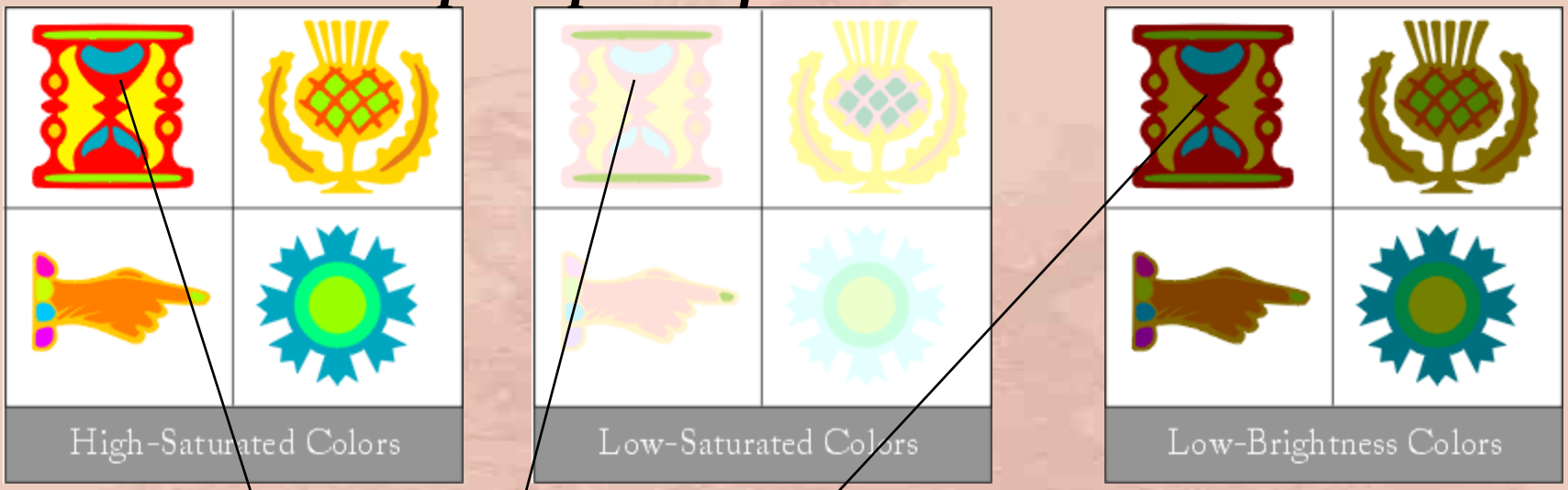
Descriptors from IA features

- **The nuclear features that most separate benign from**
- **malignant are:**

- **1.High variations and contrasts of gray levels**
- **in benign, less variations in malignant**
- **2. Peaked chromocenters more in benign**
- **3. Repetitive chromatin pattern in benign than in malignant**
- **4. Denser, darker chromatin more seen in benign**
- **5. Deviation from circularity more in malignant**
- **6. Larger shift of pixel patterns in benign**

- **These descriptors now have dimensions**

What about the issue of color images:
Human perception of color is limited.

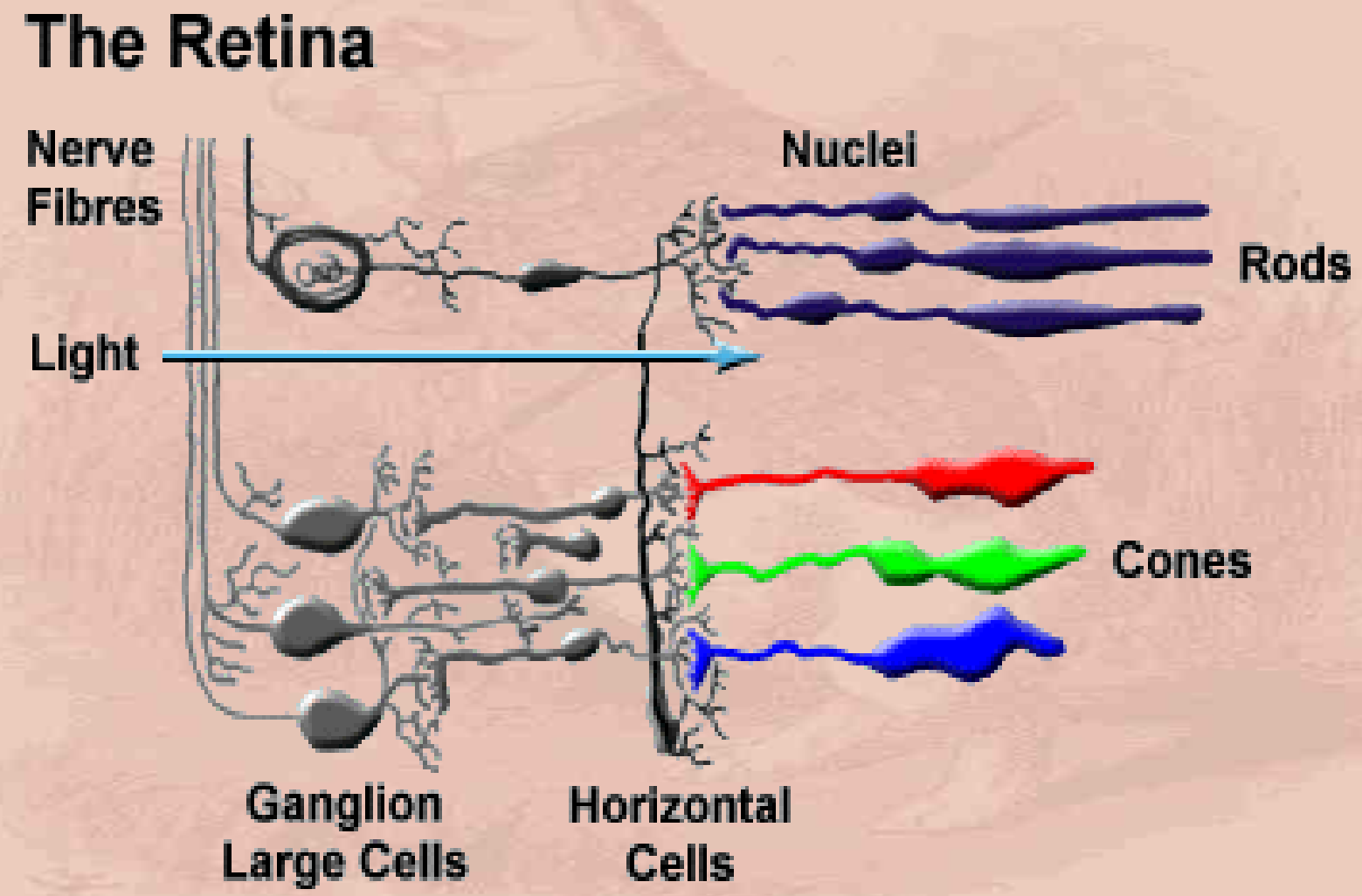


Our color visual system is influenced by the saturation and intensity component and is not equipped to handle hue invariance

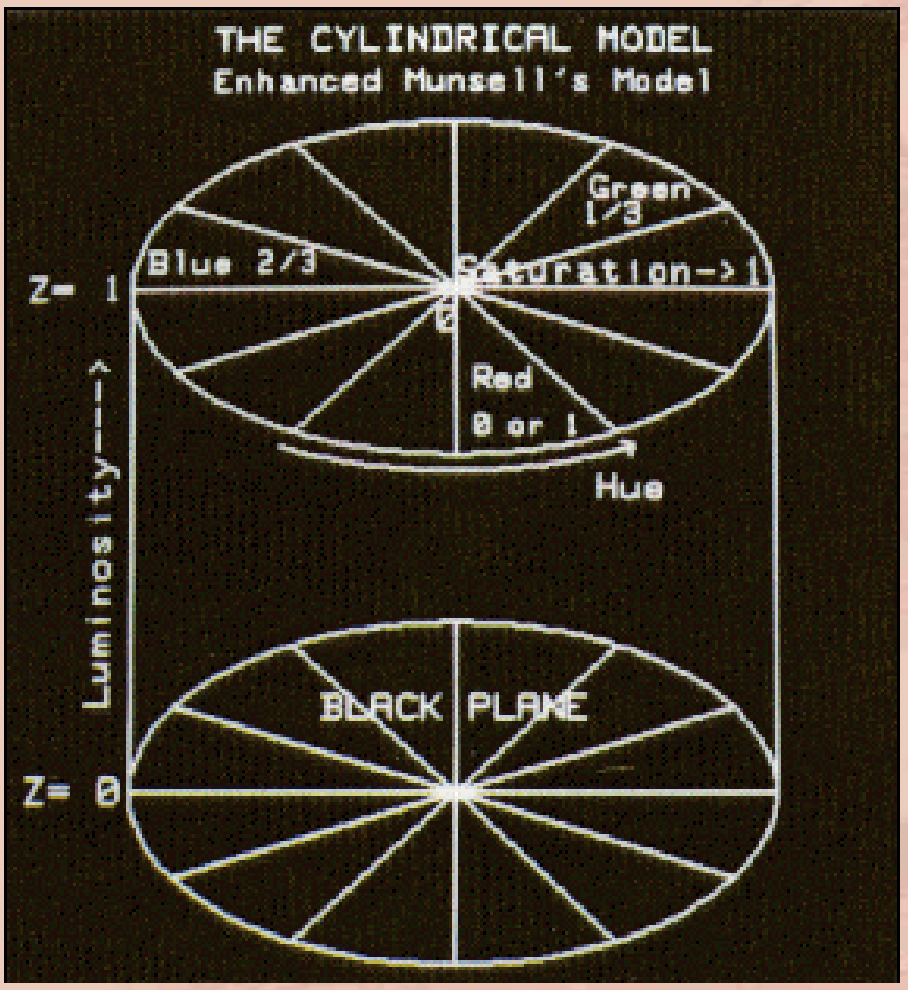
(hue = 0)

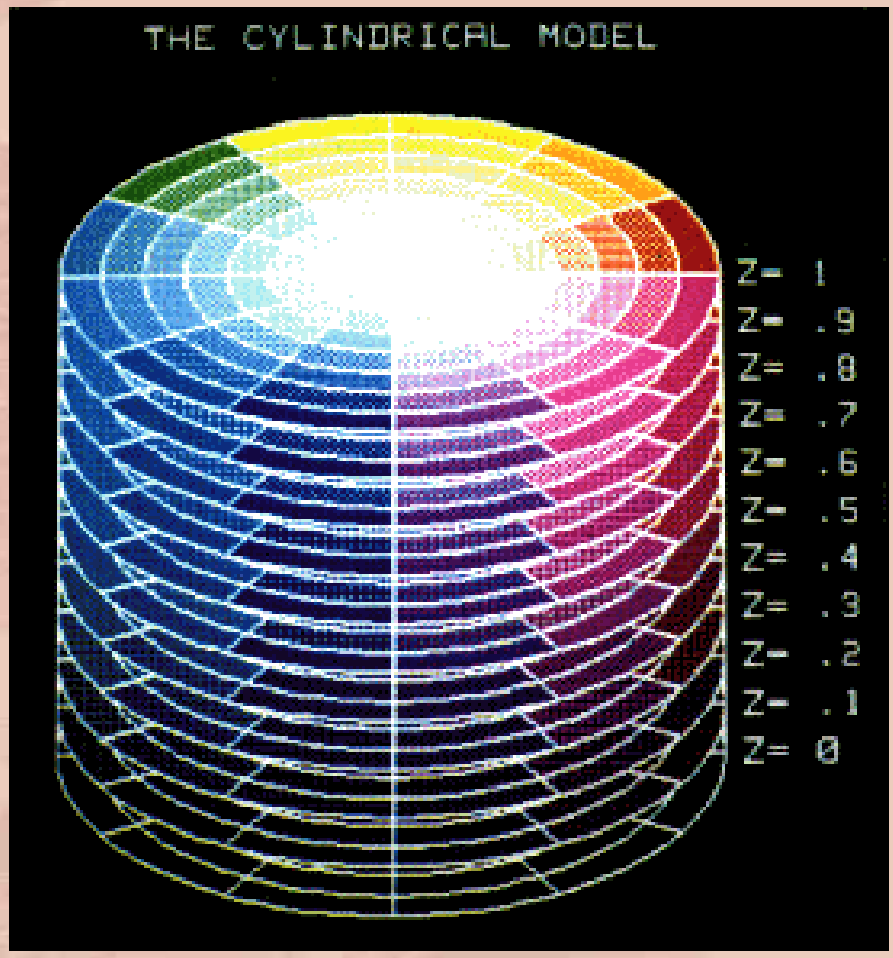
Although the hue value of the neck of the hourglass remains the same, we do not see them as equal.

Color perception and models



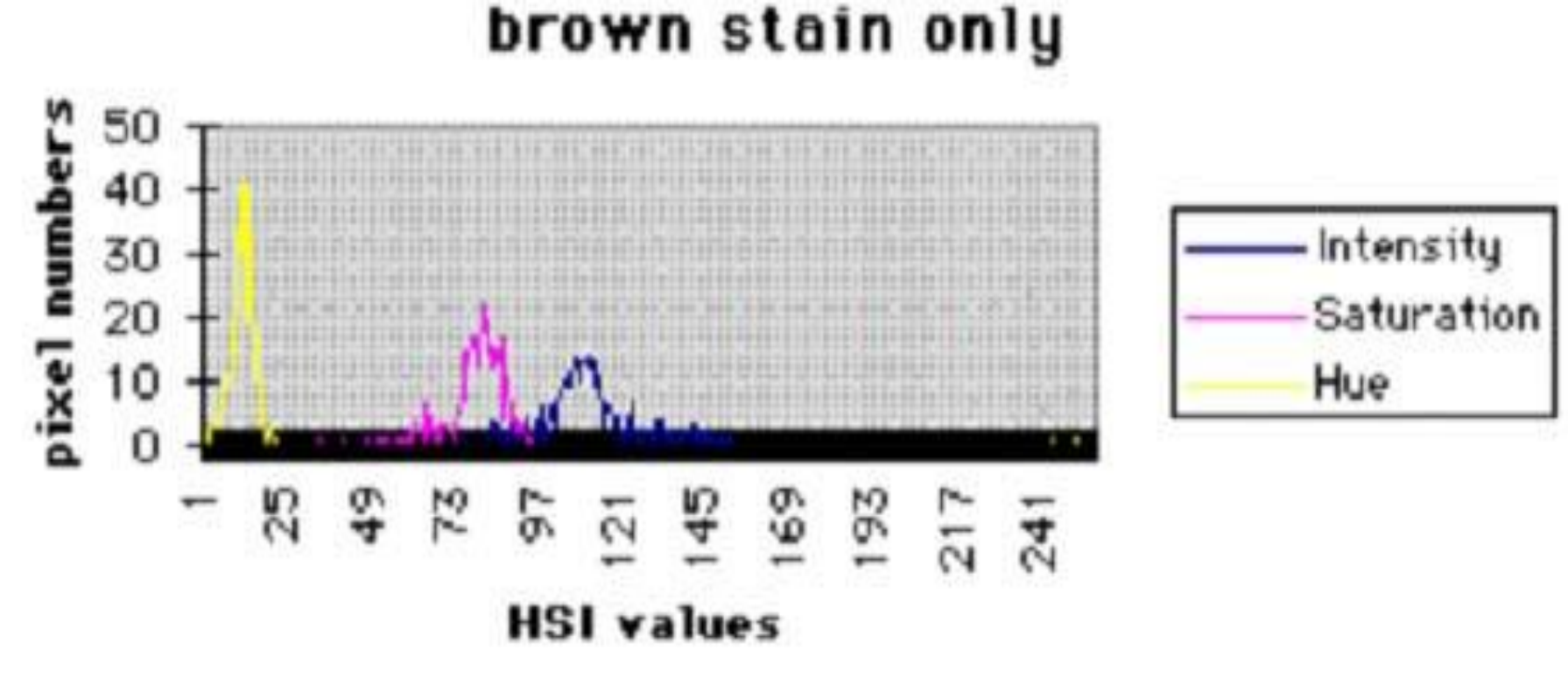
Intensity decoupled from color





A certain hue remains the same even as saturation and intensity changes

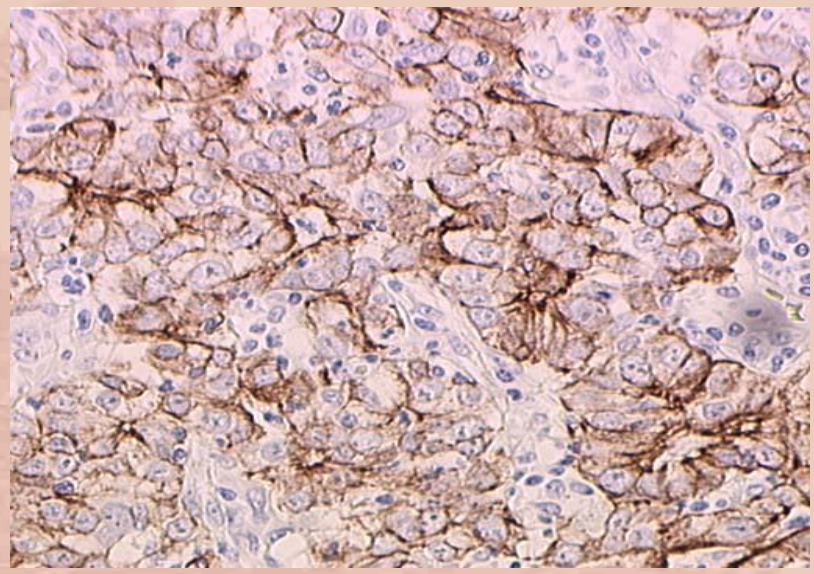
Dissociating COLOR COMPONENTS in Histology



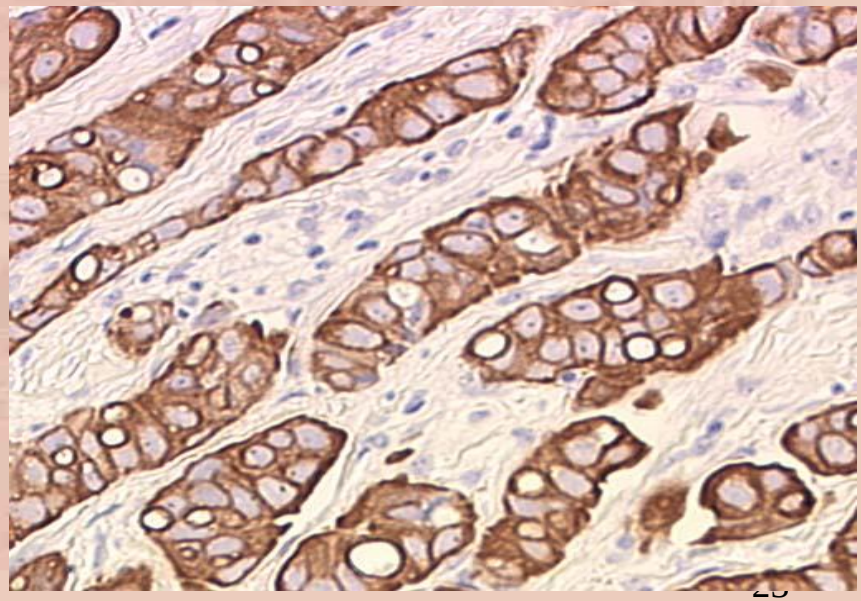
- ***histogram values could be used by thresholding algorithm to select objects from background***

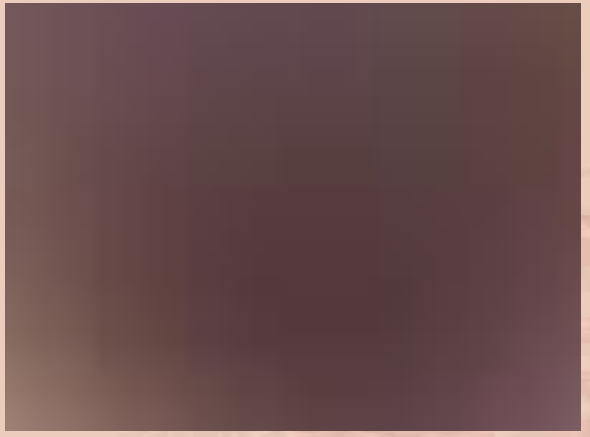
Variability of staining

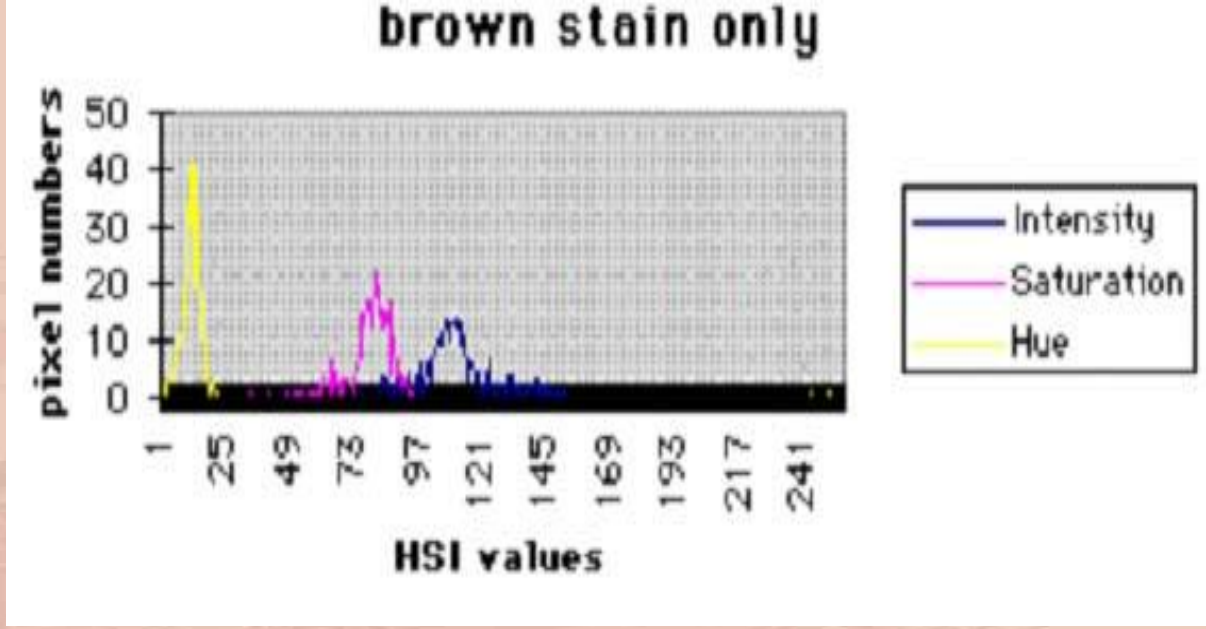
Light stained

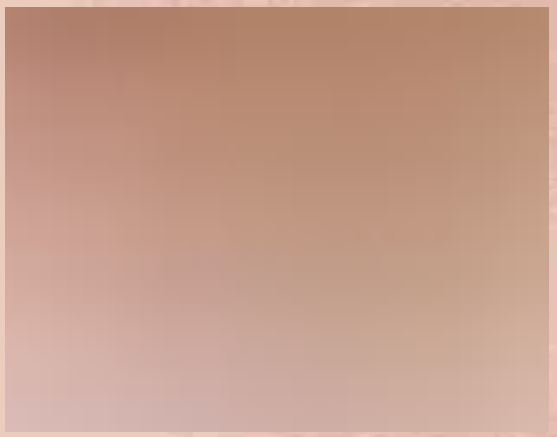


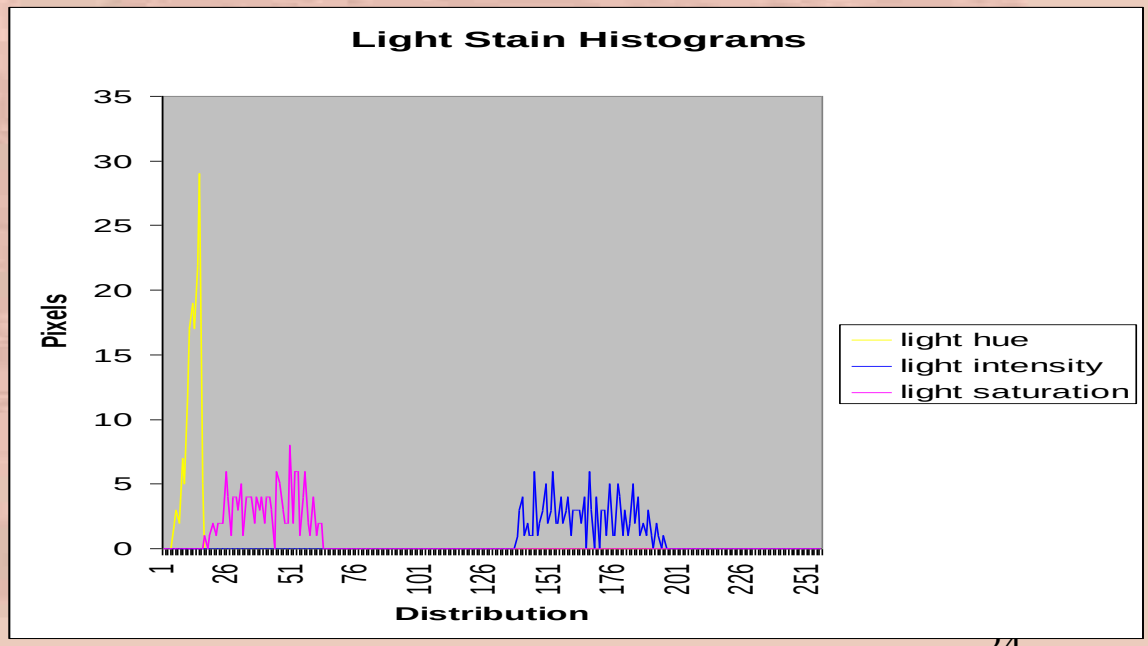
Dark stained



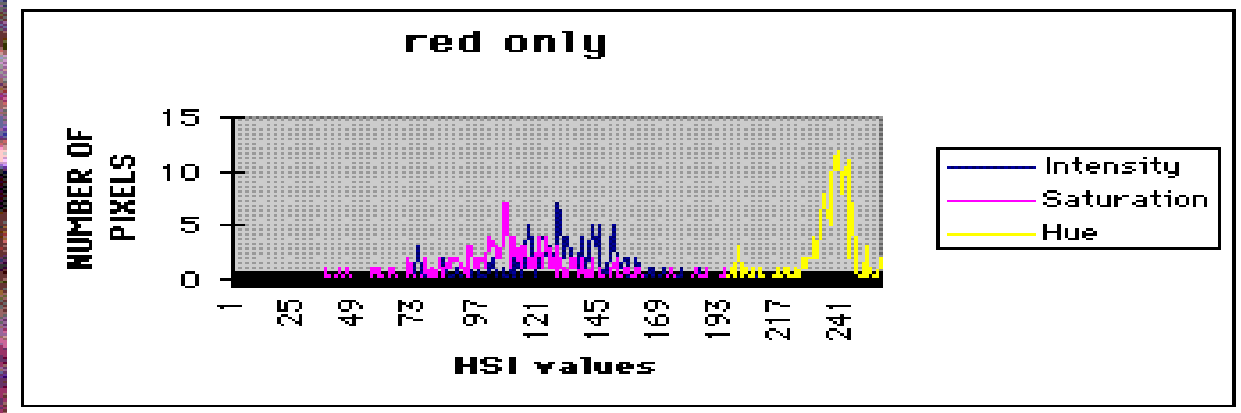


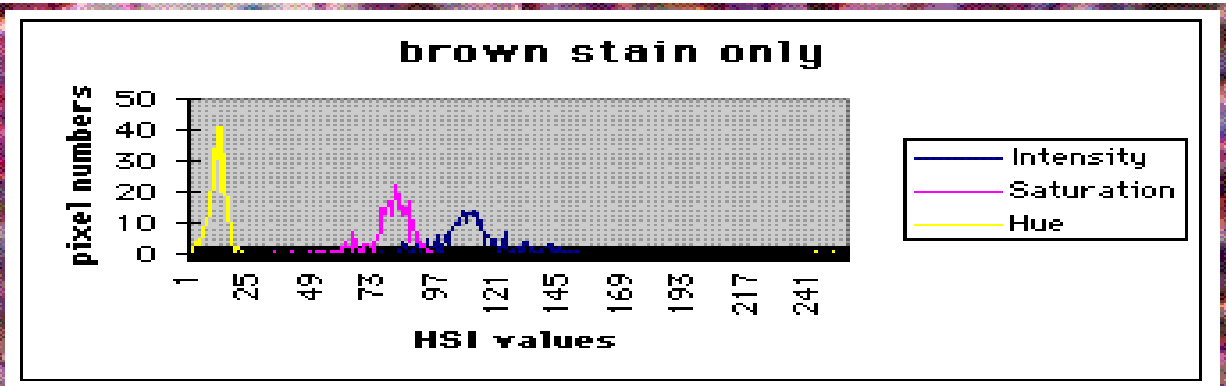

brown stain only
pixel numbers
50
40
30
20
10
0
1
25
49
73
97
121
145
169
193
217
241
HSI values
Intensity
Saturation
Hue

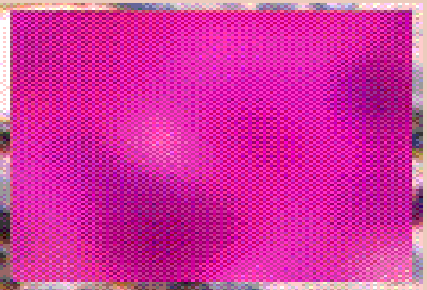



Light Stain Histograms
Pixels
35
30
25
20
15
10
5
0
1
26
51
76
101
126
151
176
201
226
251
Distribution
light hue
light intensity
light saturation

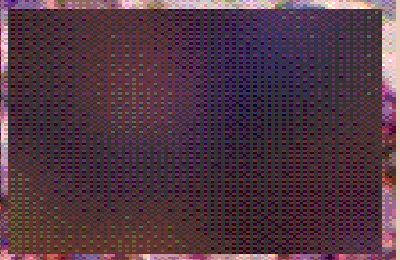
Different chromogen



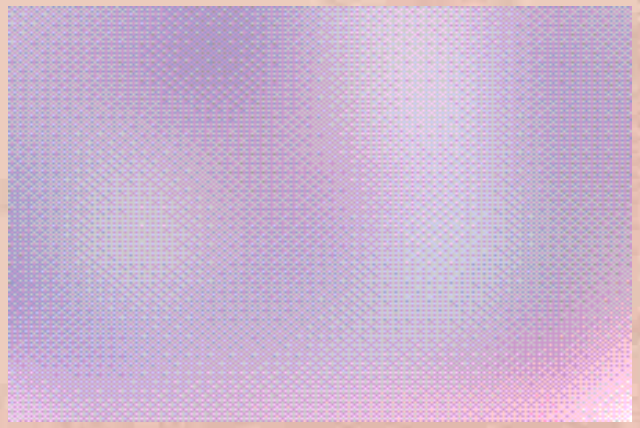


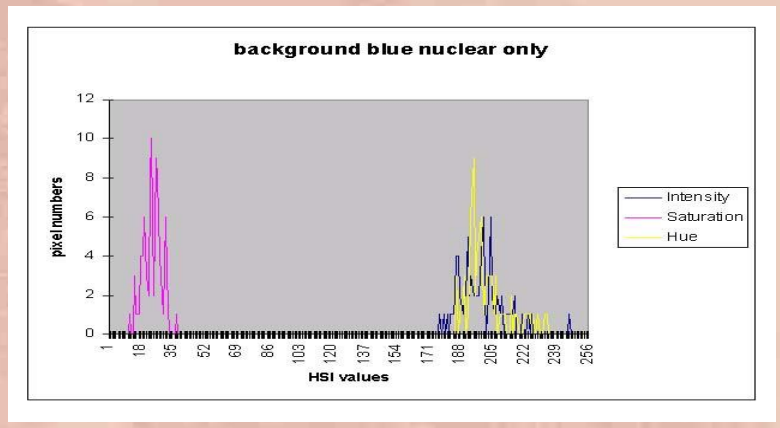


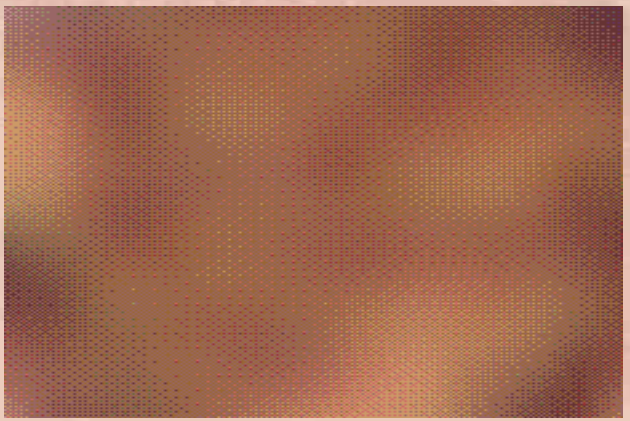
zoomed red and brown areas with respective histograms over-layed on immunohistology

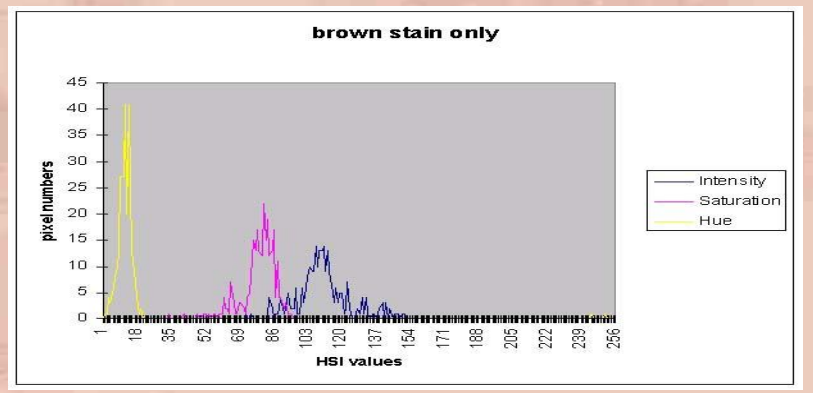


Basic color of tissue stains and their H, S, I histograms





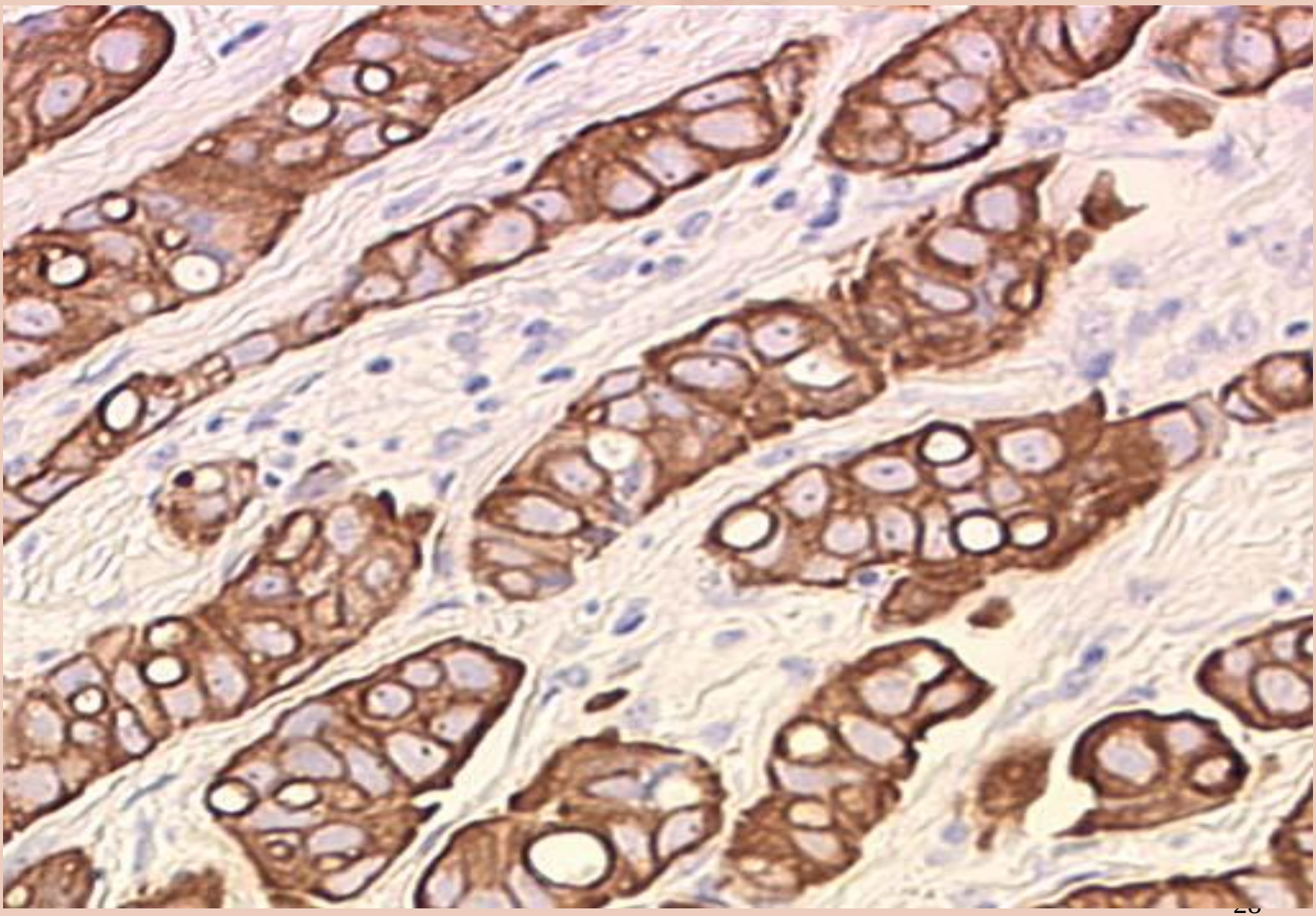


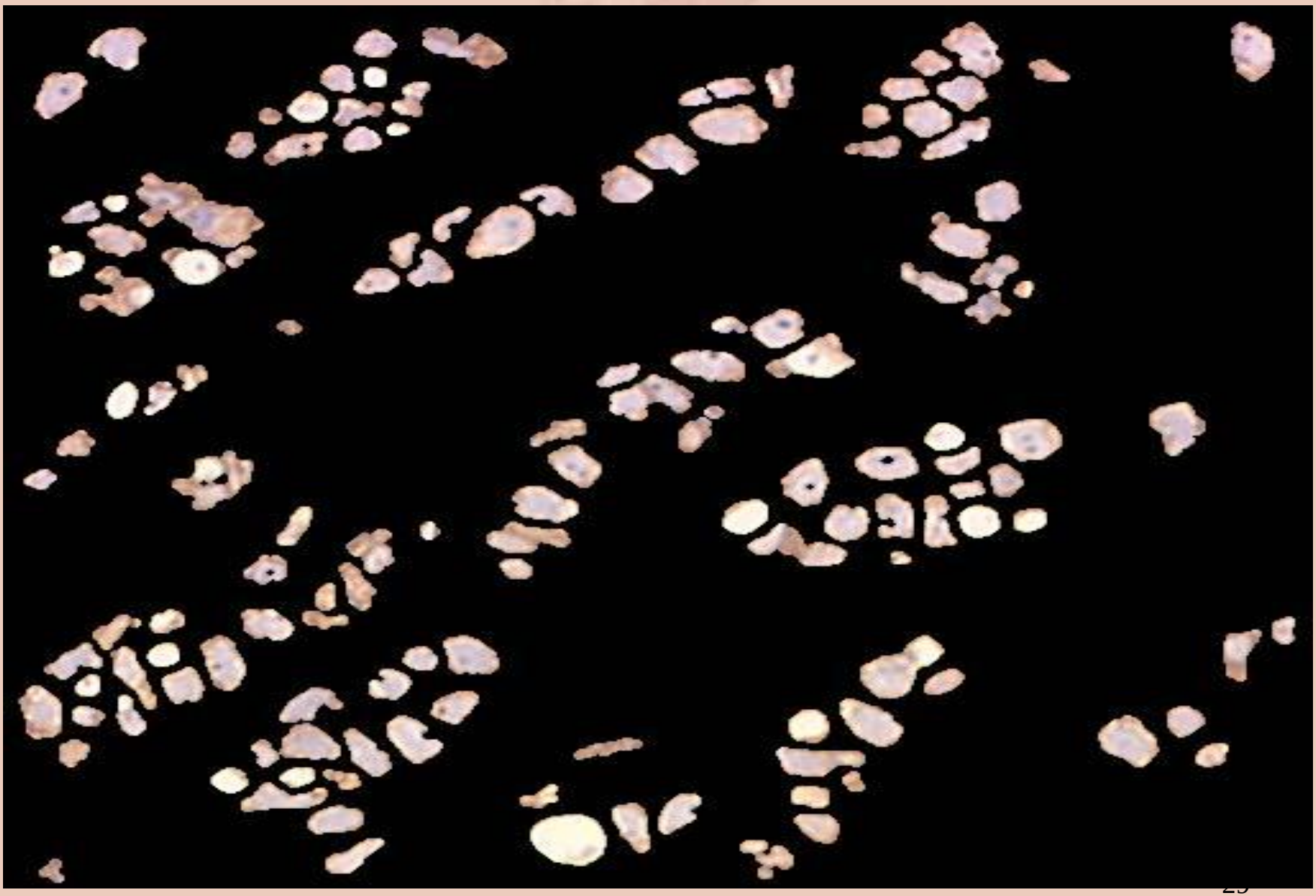


Cellular logic programming

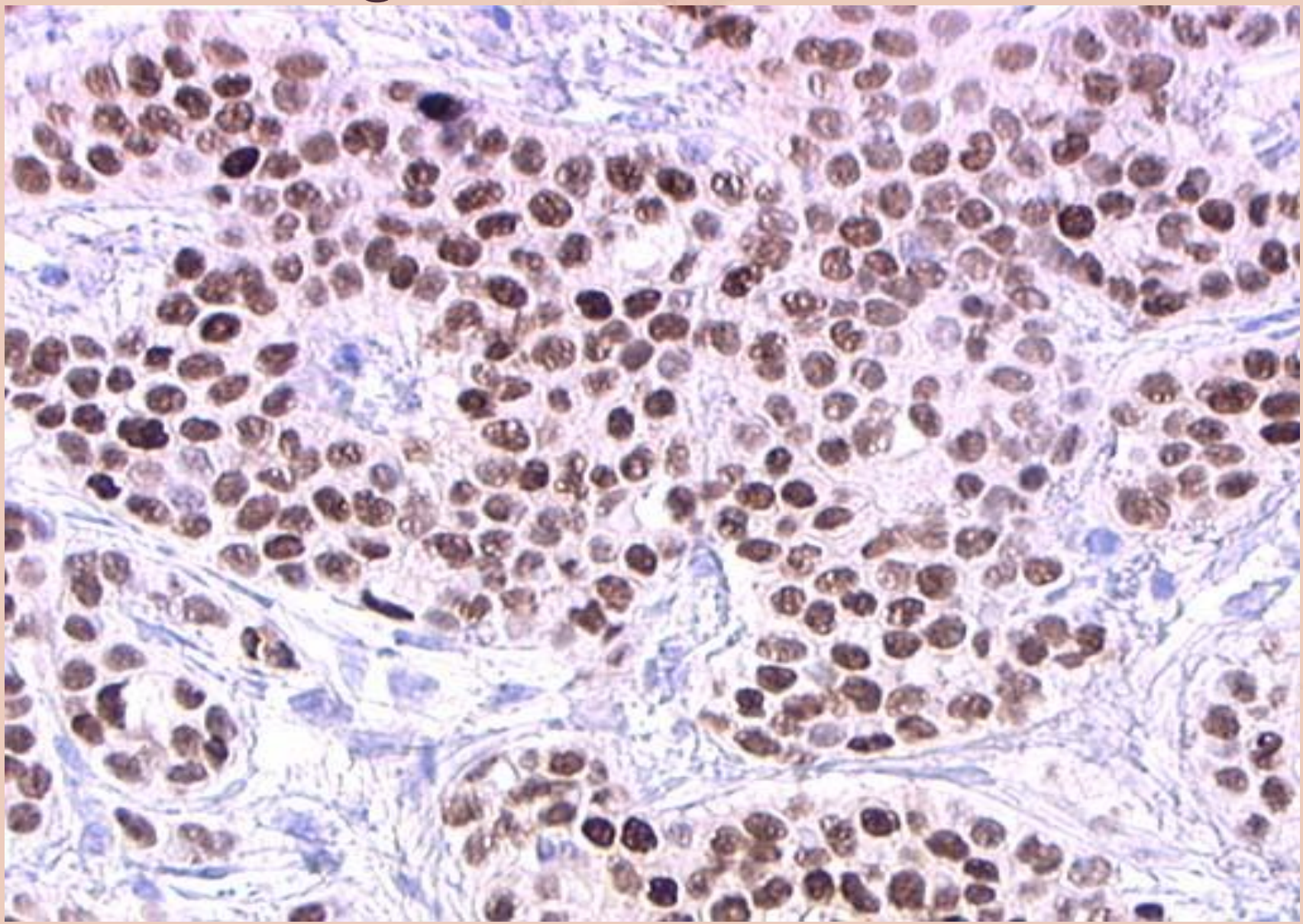
```
1.  !* three codes below apply greylevel mask to rgb source and
2.  !* get segmented result
3.  and tt at_dat_red lym1_red
4.  and tt at_dat_green lym1_green
5.  and tt at_dat_blue lym1_blue
6.  !* tt is total of ttplanes
7.  cdisp/header="objects"/viewport=5 lym1_red lym1_green lym1_blue
8.  !* temporary image for all brown stained objects without blue objects
9.  !* below to calculate density of brown and store in av_..leu_..density
10. declare bb int
11. declare average_leucocyte_density lint
12. kill/world l_stain_density*
13. declare/world l_stain_density int
14. bcount bit8 bb
15. !and cell1_green tt
16. and cell1_blue tt
17. ssum tt average_leucocyte_density
18. div bb average_leucocyte_density l_stain_density
19. sub l_stain_density 255 l_stain_density
20. !!!!!!!!!!!!!!!!!!pb l_stain_density
21. !* then get back raw area of mask
22. bcopy bit5 bit8
23. Thresh at_dat_blue bit7 e 0.01 find_thr black bit8 !e 0.16
24. band bit7 bit8
25. bdila bit8 bit8 4
26. beros bit8 bit8 1!2
```

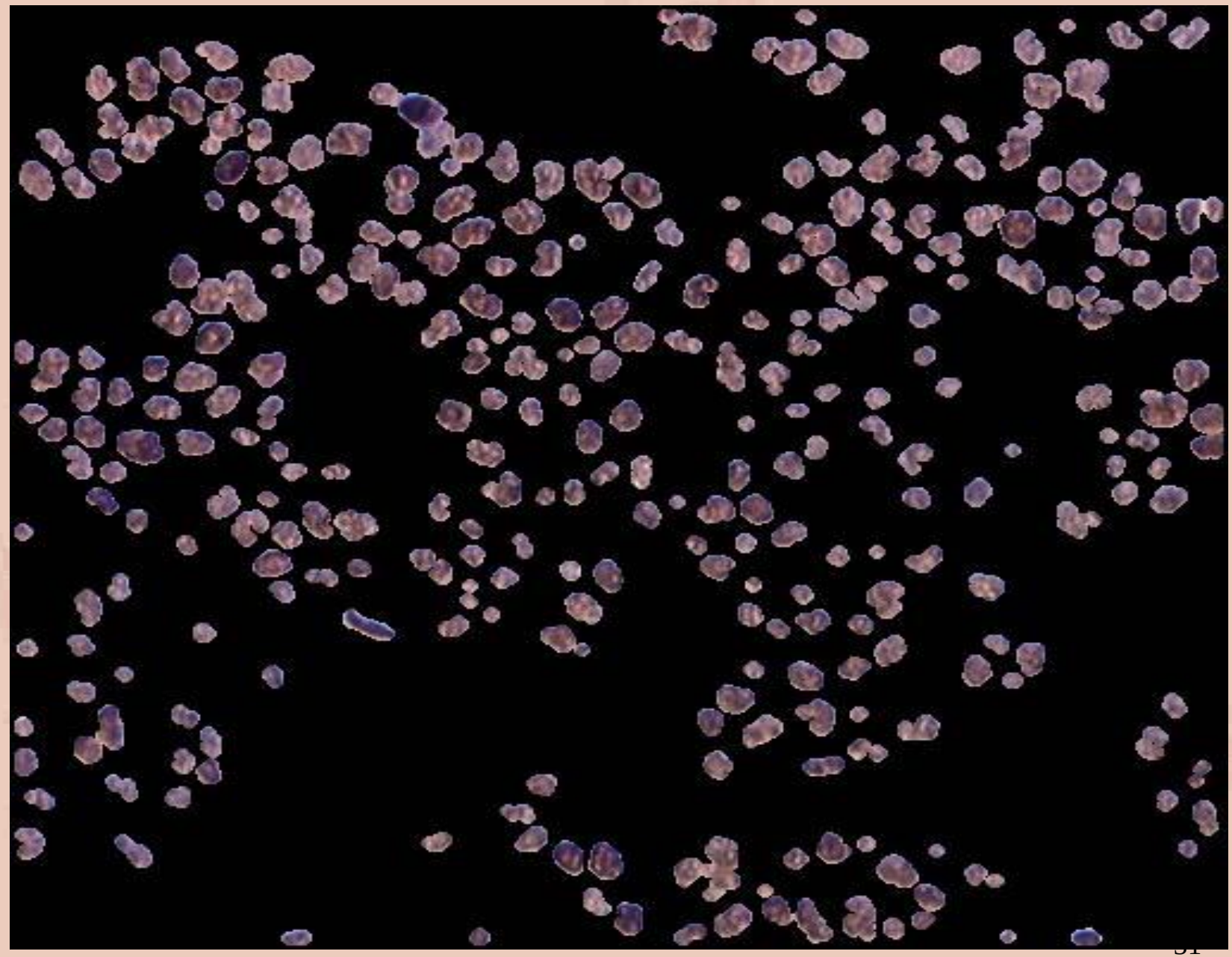
Counting carcinoma tumor burden



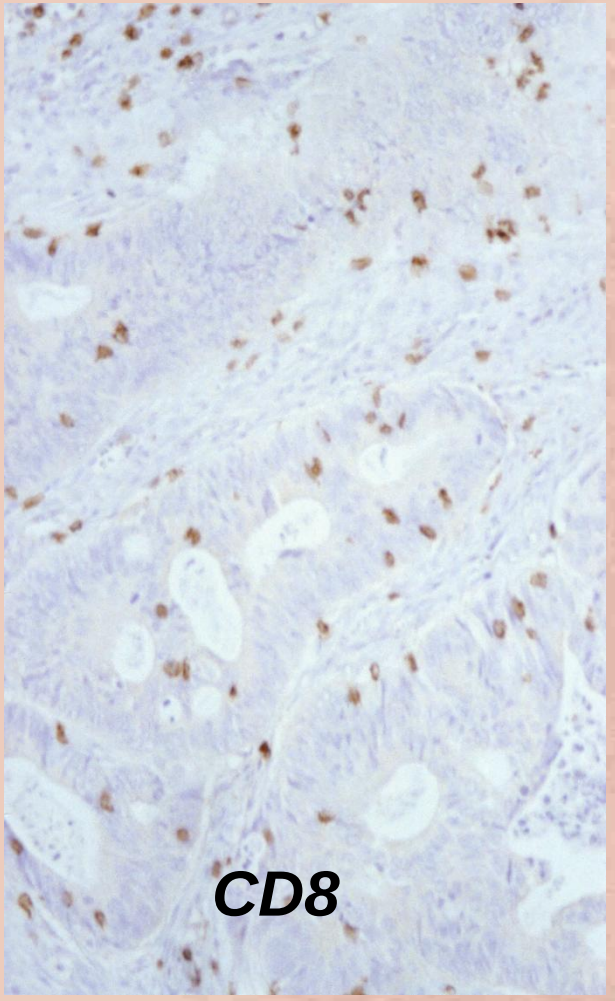


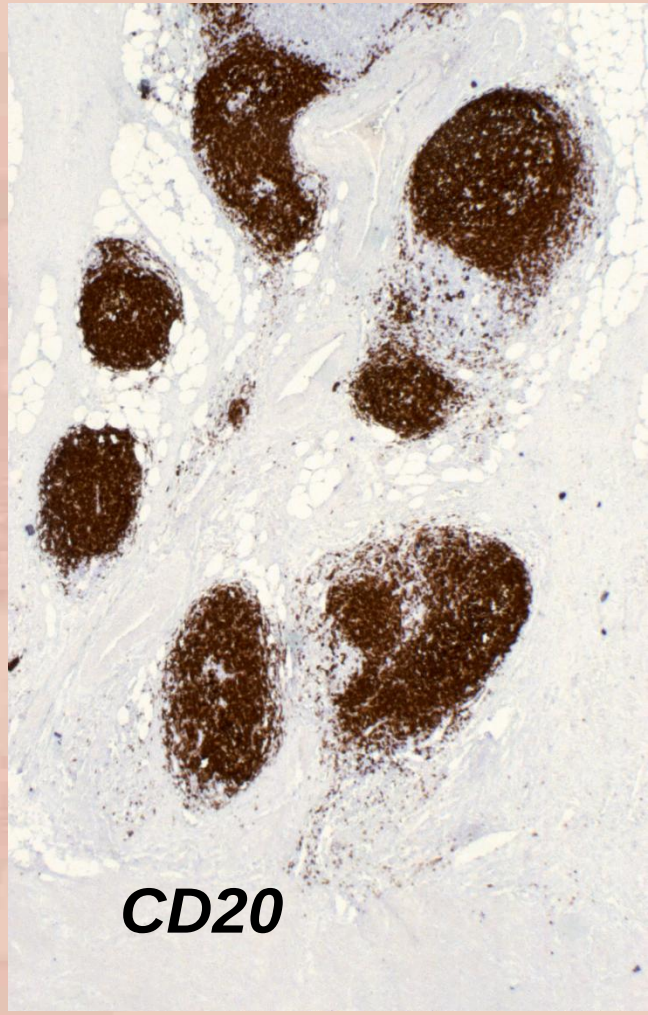
Counting nuclear stained cells

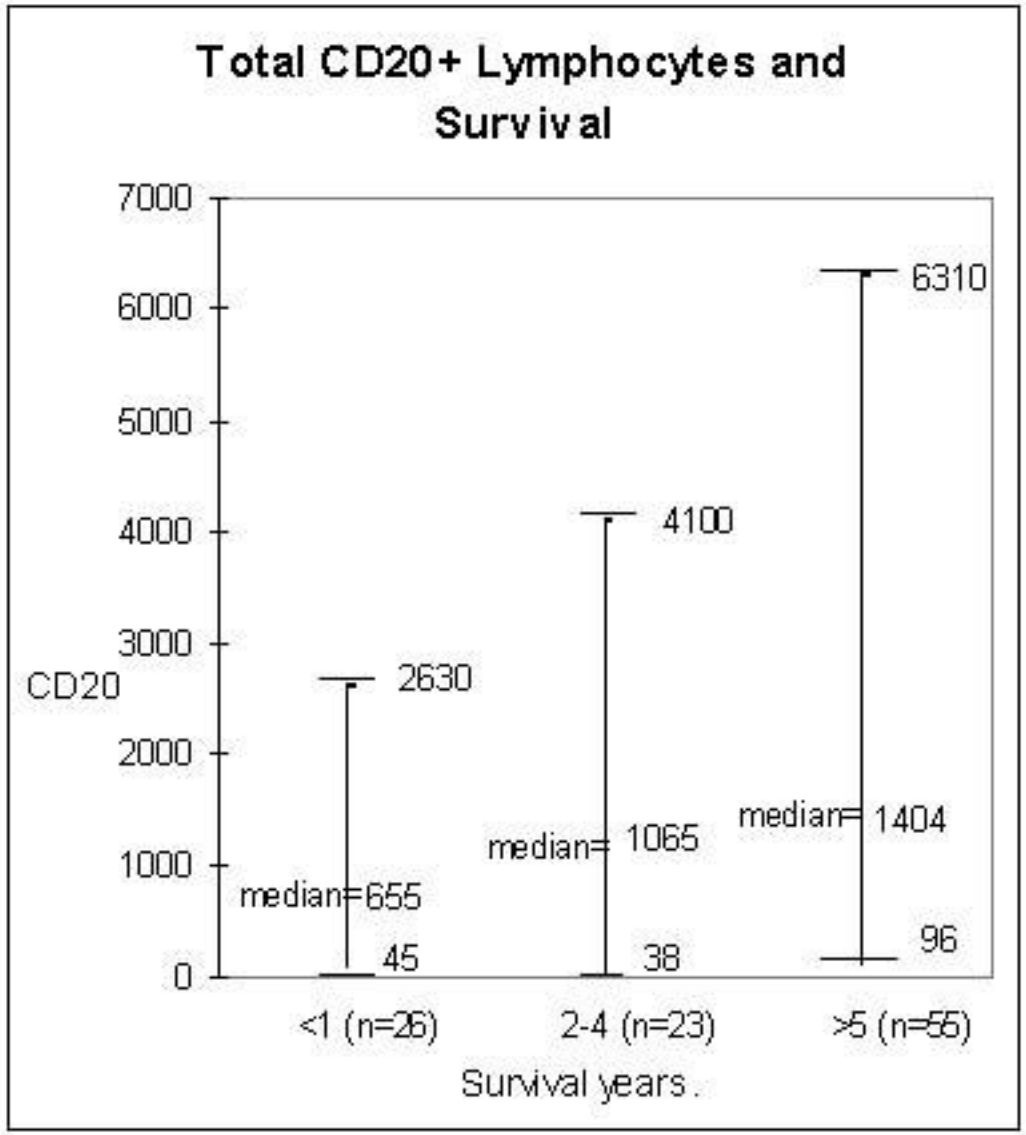


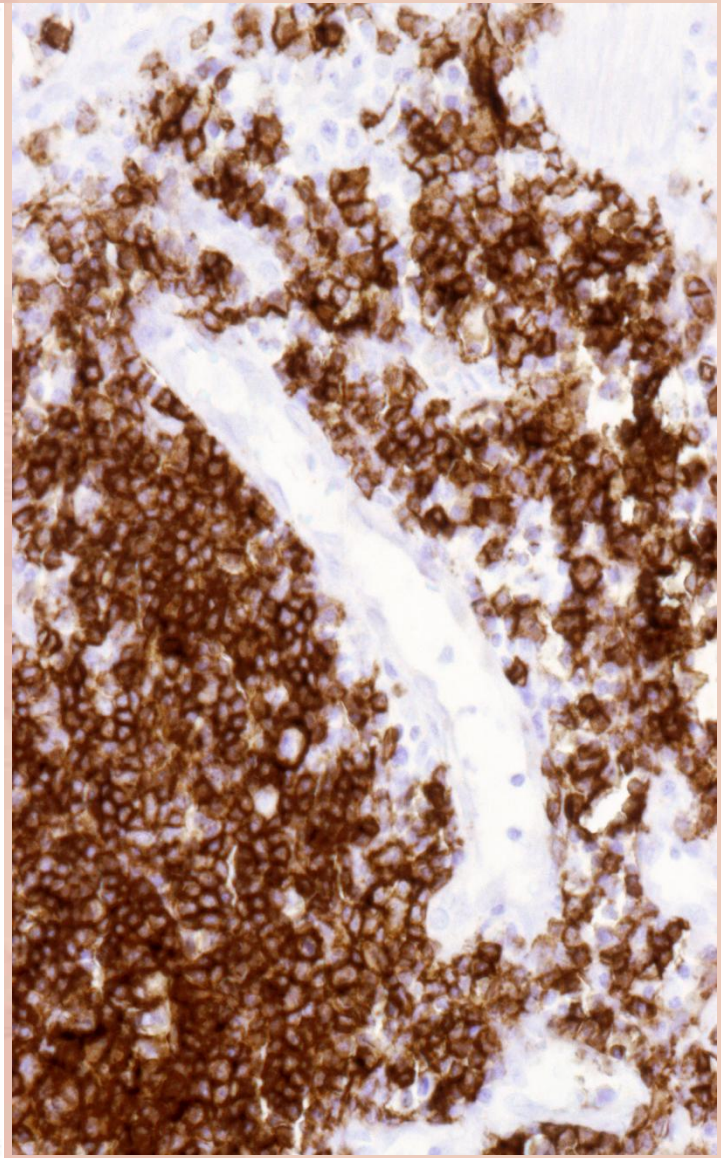


CD8 and CD20 tumor infiltrating lymphocytes (TILs) and prognosis of adenocarcinoma of the colon



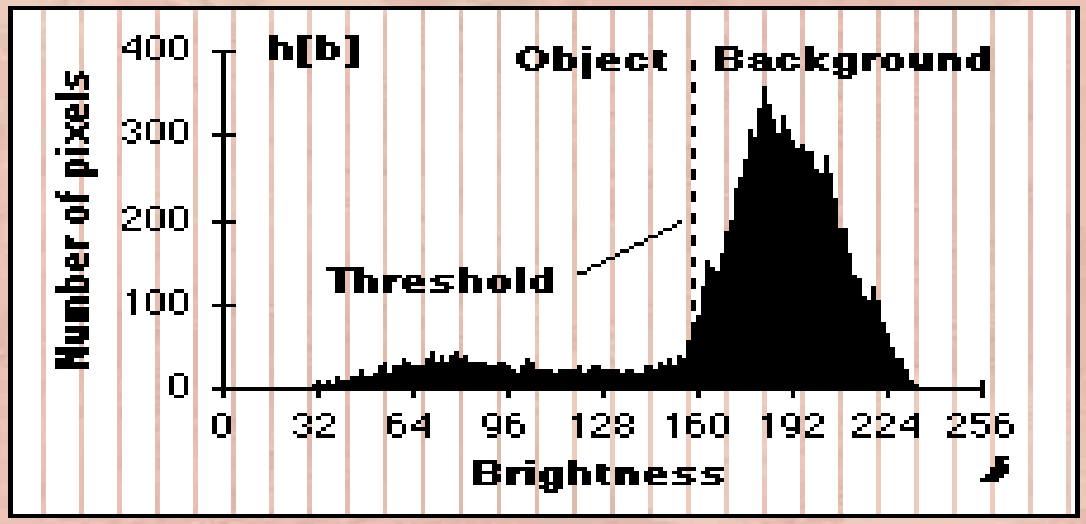


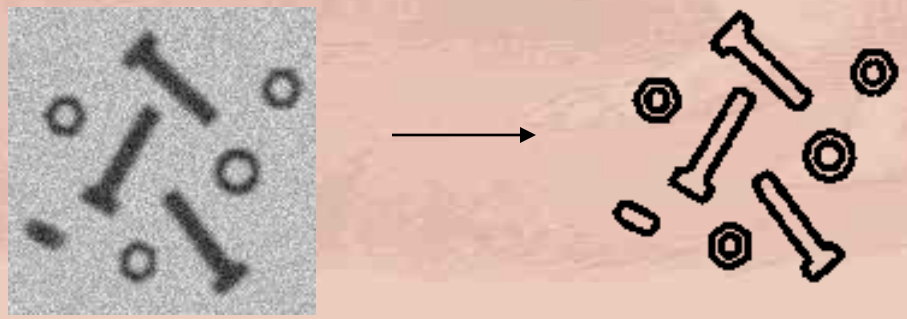

Total CD20+ Lymphocytes and Survival
7000
6000
5000
4000
3000
2000
1000
0
CD20
2630
4100
6310
median=655
median= 1065
median= 1404
45
38
96
<1 (n=26)
2-4 (n=23)
>5 (n=55)
Survival years.



Approaches to segmentation

The generic method in extracting information from an image is by segmentation- divide the image into component objects or regions. In our context, Cell Objects or COBs.





Two major methods

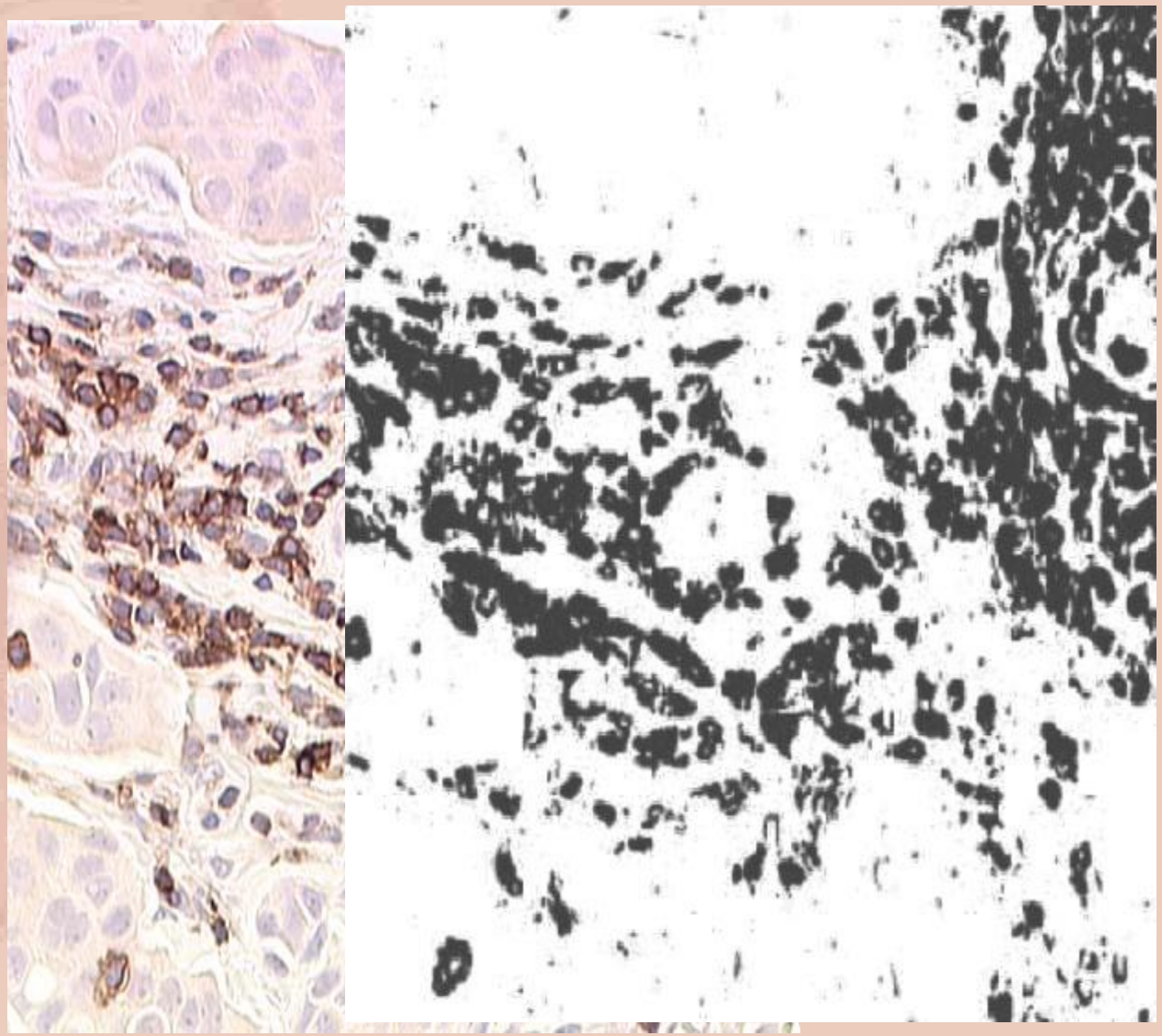
Two major approaches in segmentation use the property of color and gray level values of objects: **discontinuity and similarity**.

Objects with well-defined discontinuities usually benefit from edge-based detection and is often **pixel based** while those with poorly defined discontinuities may benefit from similarity approach such as thresholding in the **cell based or object based mode**.

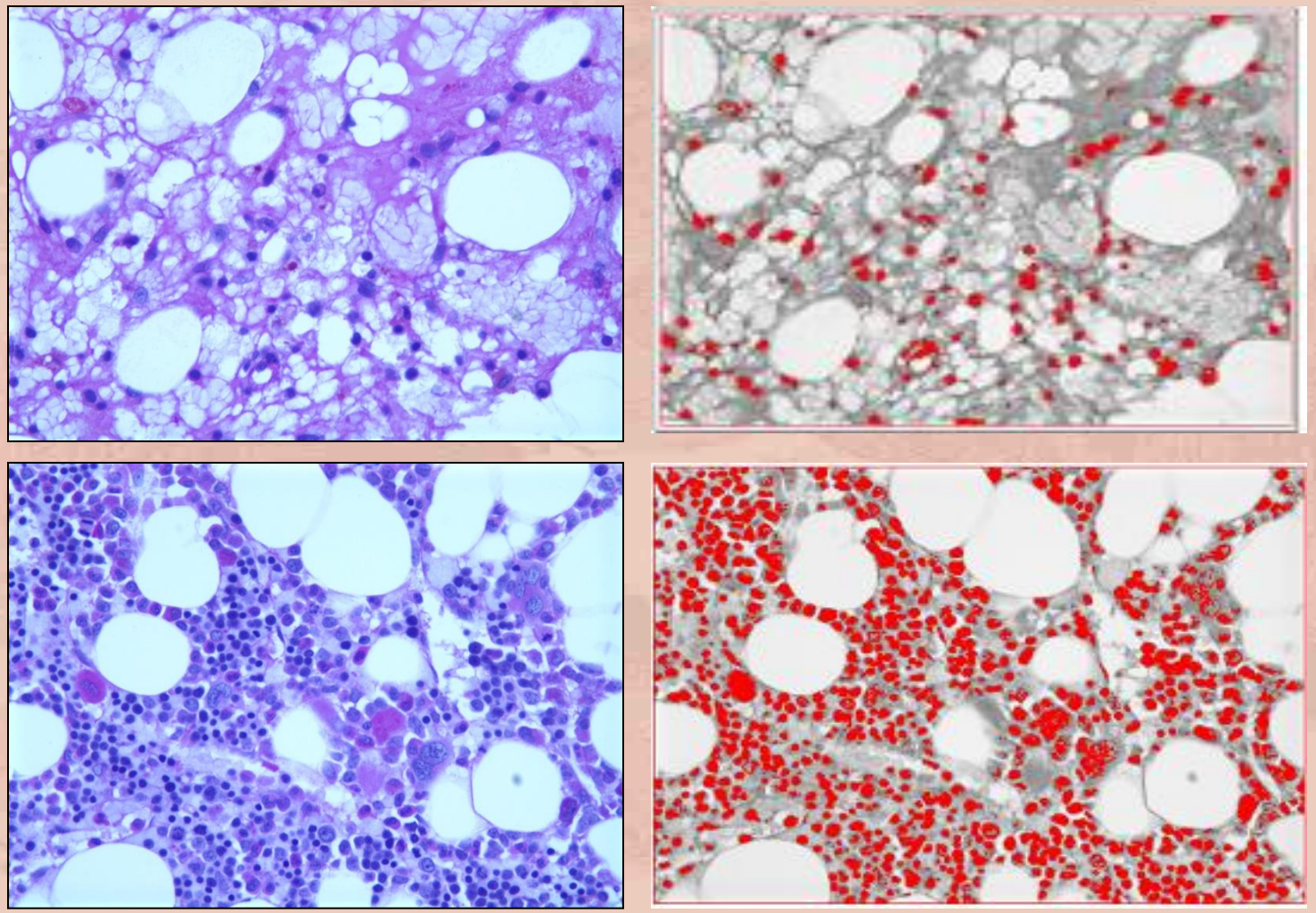
Because of the **complex, discontinuous, multicolor, large scale images** in immunohistochemically stained biologic cells, a number of studies used the **pixel mask thresholding** technic and because of similar accuracy issues have not implemented cell based reporting. Accurate segmentation remains one of the problems in pixel mask image ratio with up to 15% error on both falsely positive and falsely negative results (USPTO patent # 6,553,135). We implemented the more difficult **cell based mode.**

Approaches:

1) simple gray scale thresholding?



Simple thresholding



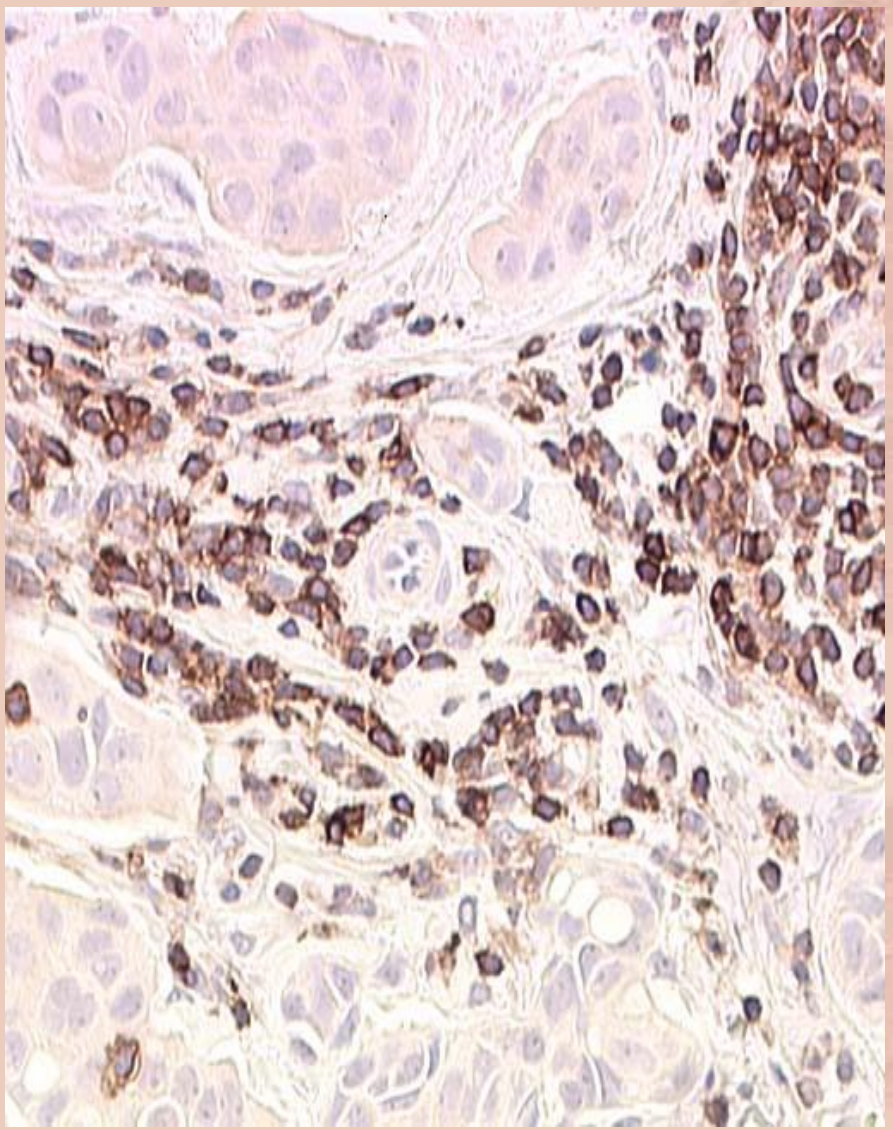
Stategy for reducing the theory of a slide cytometer or virtual flow cytometer to practice

1. Count positive and negative cells of the same cell type-phase I

2. Extract the size and color intensity information and generate a two parameter histogram distribution-phase II

3. Validate the results in relation to manual count and flow cytometry results on a defined cancer type-phase III.

Stained and unstained cells



Motivation and incentive

- The hematopathologist's standard of clinical practice in most patient's reports is to estimate the percentage of immunohistochemically stained cells and report the visual or manual estimate.
- This practice is subjective and often gives a wide range of results that depends on the level of the microscopists' skill.
- This is due to the difficulty in counting positive COBs accurately in timely manner, because of overlapped stained nuclei, variability of immunostaining, presence of other irrelevant objects, and the limitation of our visual system.
- Given the top administrative priority of minimizing medical errors in medicine in general, developing a tool in diagnostic pathology that promises similar objectivity as flow cytometry, will only decrease the incidence of errors in diagnosis.

Overlapping and variability of staining and presence of other cells of different class

Positive lymphoid cells

Negative lymphoidcells

Irrelevant objects

Non lymphoid negative tumor cells

7 Issues to solve to realize *iHCFlow* ™ Tissue Cytometry

- 1) thick tissue sections with overlapping of cells;
- 2) variability and lack of standardization of immunostaining of tissues,
- 3) optimal sampling resolution for obtaining size and staining data per cell object,
- 4) lack of a simple preprocessing technic utilizing optical output properties of image acquisition hardware,
- 5) underdeveloped cell based algorithms able to detect dual populations (positive immunostained cells and non-immunostained relevant cells of the same class) and,
- 6) lack of knowledge on standardizing threshold cut off to compensate for variability of staining and ,
- 7) lack of a cell based approach that automatically identifies the image specific parameter that optimizes detection of percentage positive cells.
- We present solutions to the above issues.

Solutions

- 1. Quality Histology
- 2. Using an automated industry standard autostainer
- 3. At 20x magnification single cell cytomics is possible
- 4. Contrast enhancement using RGB CCD blue and red filters
- 5. Above results suitable for double thresholding
- 6. Normalizing staining by percentile thresholding.
- 7. We discovered the parameters obtained from the image itself to automate the thresholding function using the entropy mode. We found a good correlation between the ratio of positive COBs with negative COBs and the value of the parameter ξ of entropy thresholding mode.

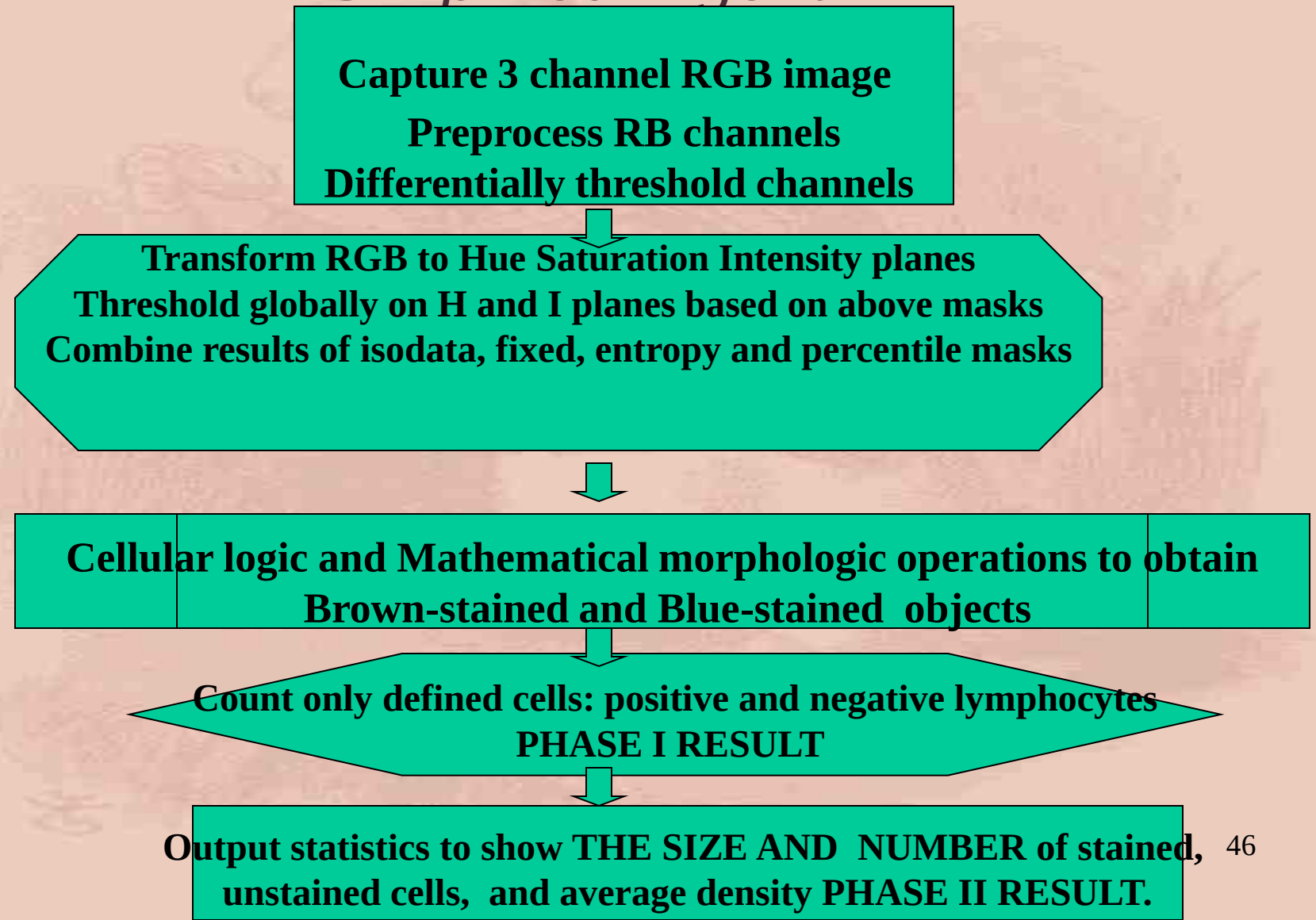
Cytomics tool

- The *i*HCFlow ™ Tissue Cytometry fulfills the major Cytomics criteria of
- 1) relating multiple parameters to each other,
- 2) within large population of cells,
- 3) on a single-cell basis,
- 4) on a quantitative and observer-independent manner.
- But unlike the other cytomics systems which use immunofluorescent tissue stains, the system differs in using routine immunohistochemistry on tissue generating flow cytometry-like results.
- *Ecker R C,Steiner GE. Microscopy-based multicolor tissue cytometry at the single-cell level. Cytometry A 2004;59:182-190.*

DESIGN AND RESULTS

- Segmentation of a 512 x 474 RGB image and tabular display of statistical results table took 12 to 15 seconds using proprietary developed algorithms. We used a panel of 7 antibodies for validation on 14 cases of mantle cell lymphoma giving percentage positive, total lymphocytes, and staining density. A total of 2,027 image frames with 810,800 cell objects (COBs) were evaluated. Antibodies to CD3, CD4, CD8, Bcl-1, Ki-67, CD20, CD5 were subjected to virtual flow cytometry on tissue. The results of Tissue Cytometry were compared with manual counts of expert observers and with the results of flow cytometric immunophenotyping of the same specimen.
- The correlation coefficient and 95 % confidence interval by linear regression analysis yielded a high concordance between manual human results (M), flow cytometry results (FC), and Tissue Cytometry (TC) results per antibody, ($r = 0.9365$ manual vs TC, $r = 0.9537$ FC vs TC). The technical issues were resolved and the solutions and results were evaluated and presented.

Simplified Algorithm



iHCFlow ™ Tissue Cytometry

- A method and approach is developed for fully automated measurements of immunostained lymphocytes in tissue sections by means of digital color microscopy and patent pending advanced cell analysis.
- The validation data for population statistic measurements of immunostained lymphocytes in tissue sections using Tissue Cytometry is presented.
- The report is the first to describe the conversion of IHC data to a flow cytometry-like two parameter dot-plot display, hence the technic is also a virtual flow cytometry.

How we differ from previous studies

- Previous studies have reported on using spectral capable instrumentation and wavelength filters, confocal immunofluorescent microscopy to obtain separation of positive stain from nonstained pixels. Also a few hybrid microscope-flow cytometry were developed to obtain COBs information to mimic flow cytometry. Immunofluorescent technics were applied on slide substrate to obtain cell populations using laser scanning instrumentation.
- The bandwagon technic is pixel-counting using ACIS and others using Hot spots paradigm to sample IHC stained tissue using single target markers Herceptin, EGFR, BcrABL-
- We believe our approach does not need the complex, fluorescent and expensive instrumentation not readily available to a regular pathologist or oncologist.
- Few studies looked at counting immunohistochemistry stained slides, but none converted the results to a flow cytometry paradigm.

Pixel counting and Cell based counting

- *Two major approaches in counting the positively immunostained population in tissue are pixel (area) based or COBs (population) based methods. Most of the previous studies on counting immunohistochemical positive COBs are pixel based(34,35,32,21,36)(Brey et al. 575-84;Brey et al. 279-94;Johansson et al. 1073-79;Ornberg 1059-60;Sont et al. 1496-503).*
- *Population based image analysis is more accurate, intuitive, and similar to the flow cytometry paradigm. Population based analysis is more difficult and challenging than pixel based technics and only a few studies are seen (37,13,21,28)(Dahle et al. 182-88;Loukas et al. 30-42;Ornberg 1059-60).*
- *We have implemented a population based image analysis with good results. Most of the previous approaches used pixel data to obtain the percent positive COBs or quantity of antigen present.*

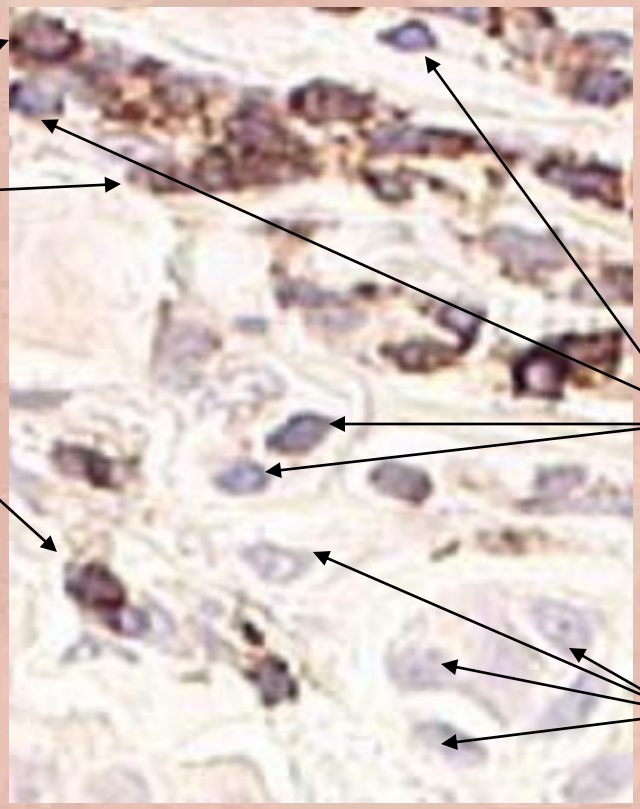
Prior art

- Flow Cytometry method uses fluorescence and laser light scattering methods, and then analyzes with powerful graphical software tools to obtain meaningful population-based information.
- The laser scanning cytometer - a cell based system using segregated cells in a proprietary cell well substrate. It does not perform on a regular tissue stained immunohistochemistry slide. The current commercial image analysis systems will not perform cell based brightfield cell analysis in routine immunostained slides.
- The Chromavision ACIS could do many slide based analysis but has limited the population statistic analysis to getting pixels that are positively stained over all the area of the frame and has in its patent allowance for a 15% error on both falsely positive and falsely negative results. As much as 15% error on both falsely positive and falsely negative results is published (USPTO patent # 6,553,135). A number of approaches used the pixel mask technology and because of similar accuracy issues have not implemented population statistic reporting. Similar to Ventana VIAS.
- The ARIOL system of Applied Imaging uses automated slide delivery to microscope and perform similar capacities as the Chromavision. It has been using the pixel mask technology and because of similar accuracy issues has not implemented its population statistic reporting.
- PAXIT systems has a limited module to do population statistics but only appears to count the positively stained cells in a nuclear pattern and is not validated or widely used.

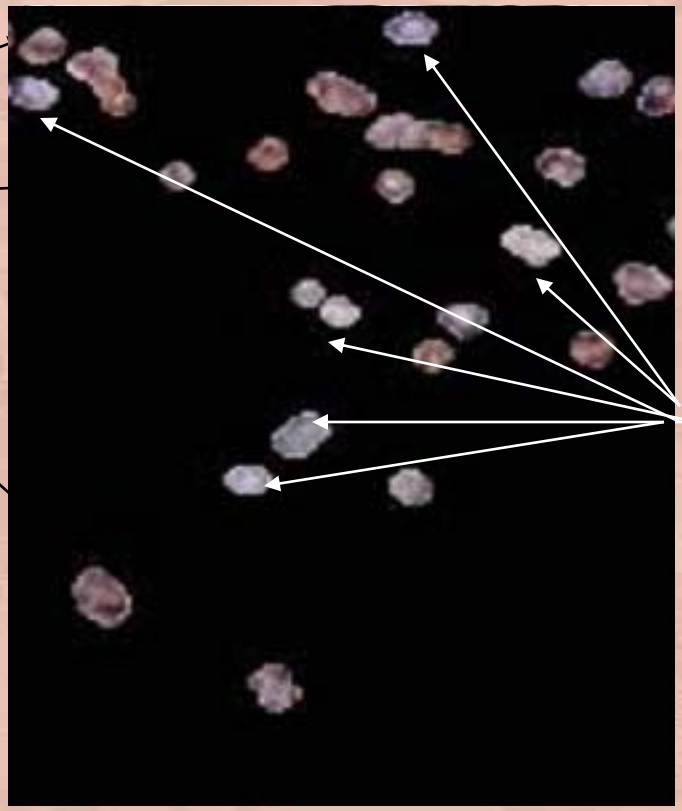
Results of Phase I Study : CD8 Tumor infiltrating Lymphocytes with Positive and Negative Cells

	ImmunostainDensity	Lymphocyte Number	Case x Lymphocytes (+) stain	Lymphocyte stain (+) (%)
1	4	58	23	39.655174
2	4	52	22	42.307693
3	4	57	43	75.438599
4	4	56	22	39.285713
5	4	61	44	72.131149
6	4	106	48	45.28302
7	4	54	25	46.296295
8	4	74	13	17.567568
9	4	43	33	76.744186
10	4	45	22	48.888889
11	4	36	18	50
12	4	73	8	10.958904
13	4	131	13	9.923664
14	4	86	24	27.906977
15	4	42	18	42.857143
Min	4	36	8	9.923664
Max	4	131	48	76.744186
Median	4	57	22	42.857143
Total		974	376	
Average	4	64.933334	25.066668	38.603695

Raw color frame for manual interpretation



Example of advanced thresholding to obtain phase I result



Phase II extraction of cell objects data

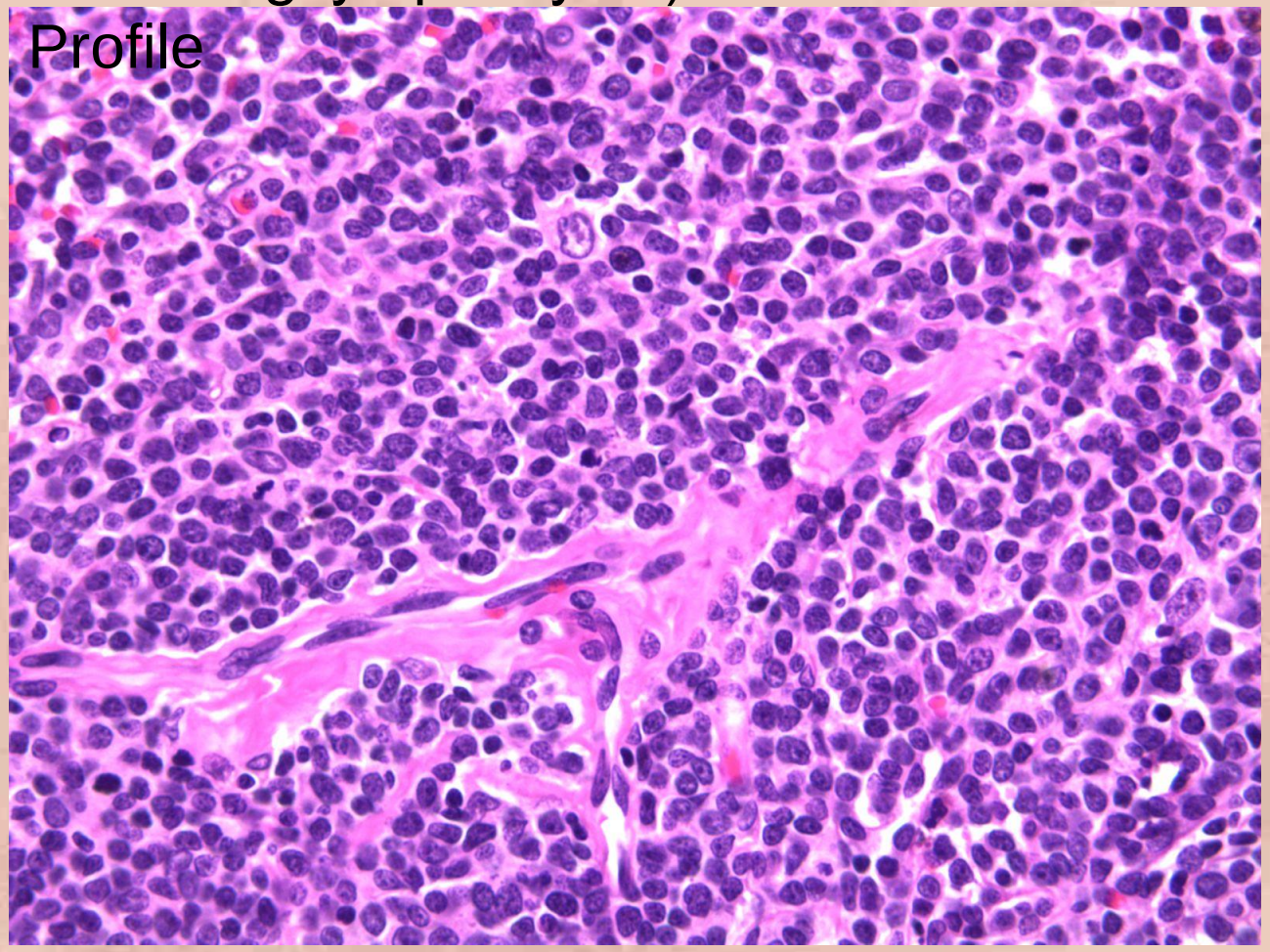
- The formula below was used to convert pixel area to cell diameter in microns (Y data), for a 20x magnified image:

Cell diameter = 2 * (sqrt of (Area in pixels / π)) / 1.5

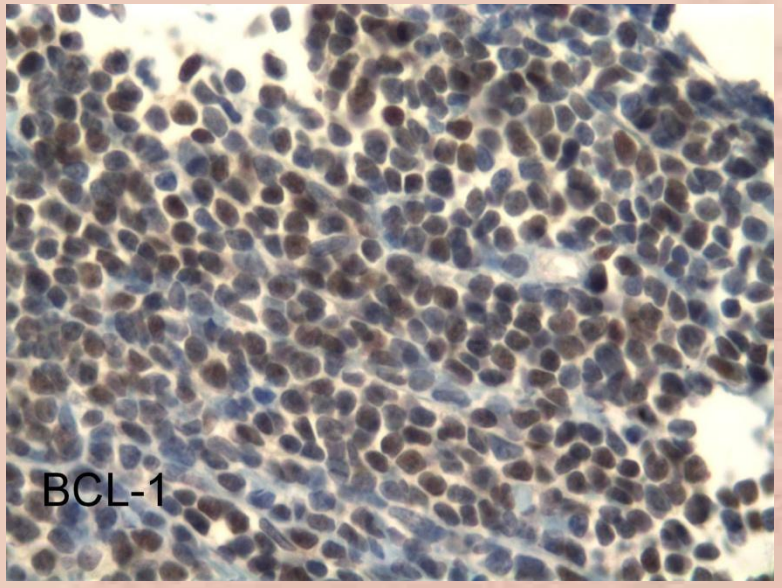
= sqrt of (Area in pixels * P) ;where diameter is in microns, P is conversion factor (0.56588424212)

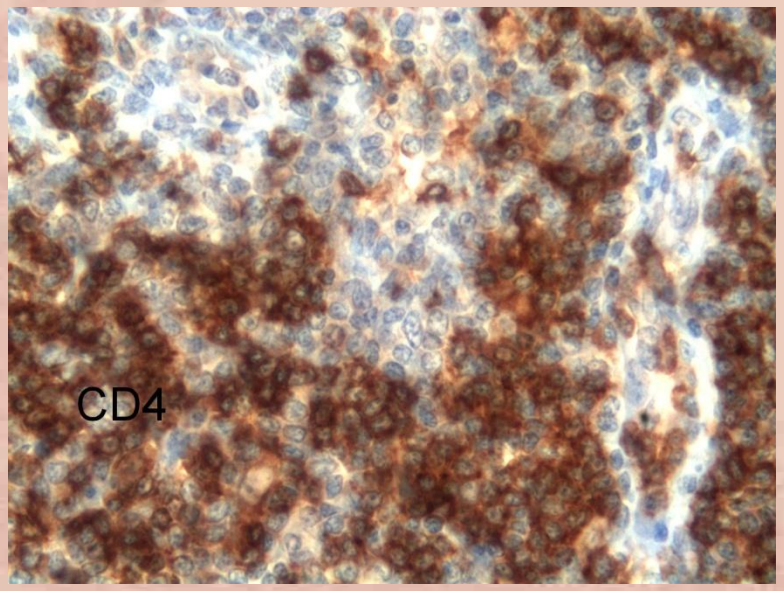
- The optical density data per cell object was given by the sum of optical density of the colored pixels comprising the previously labeled COBs (X data).
- Each cell object then was labeled and contained size and density numbers which were used for the dot plot display COB. The final resulting data then consisted of an array of labeled objects with size and corresponding stain density.
- These two parameters defined the frequency distribution of the COBs in the two dimensional dot plot.

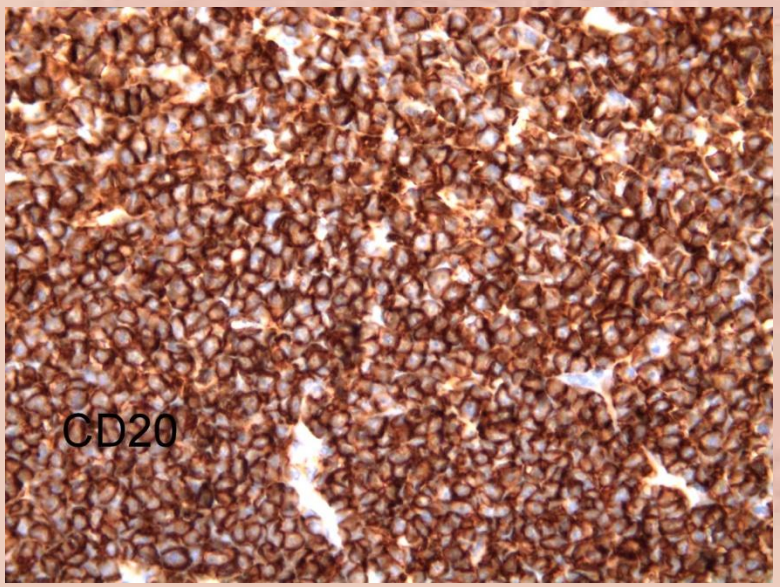
VALIDATION OR PHASE III: PROJECT on MANTLE CELL LYMPHOMAS AND (Tumor Infiltrating lymphocytes) TIL'S and Marker Profile

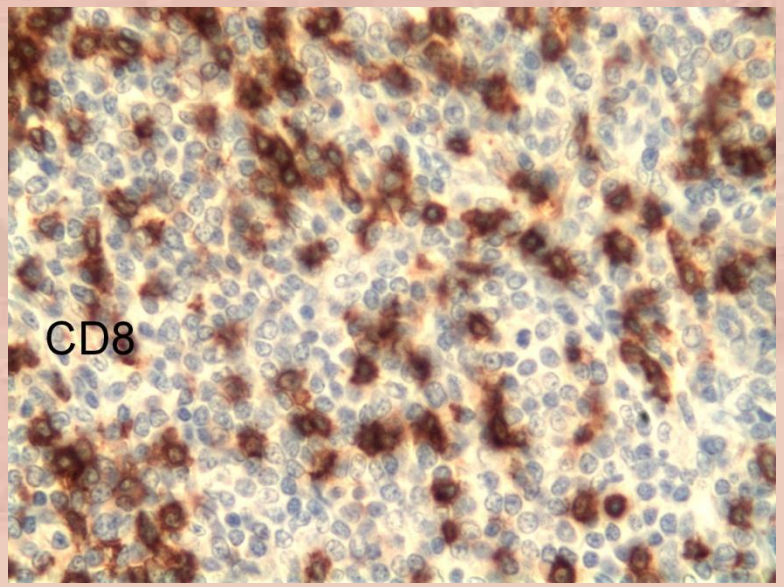


Mantle cell lymphoma marker profile and TIL's







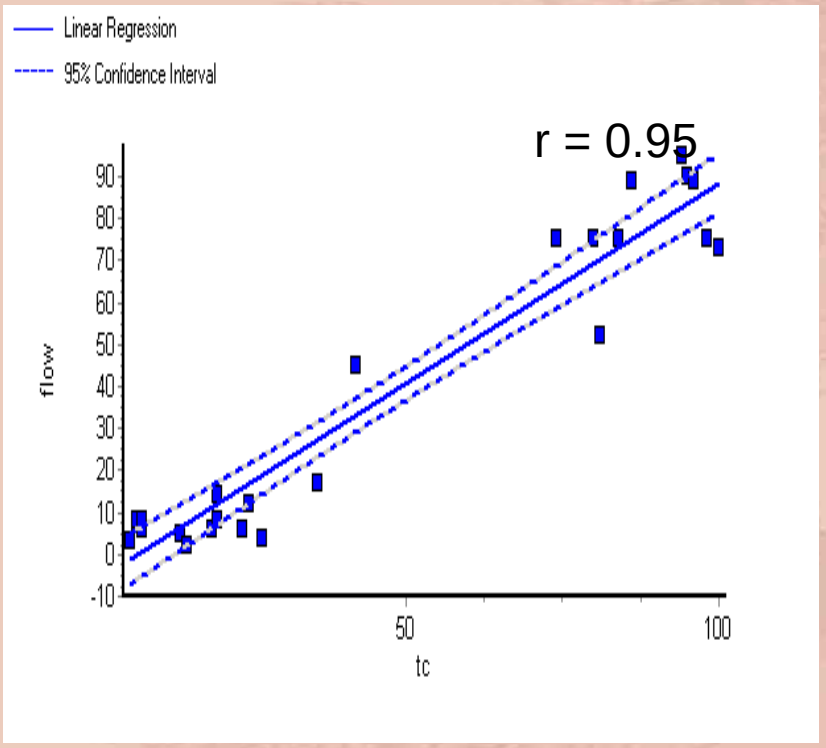


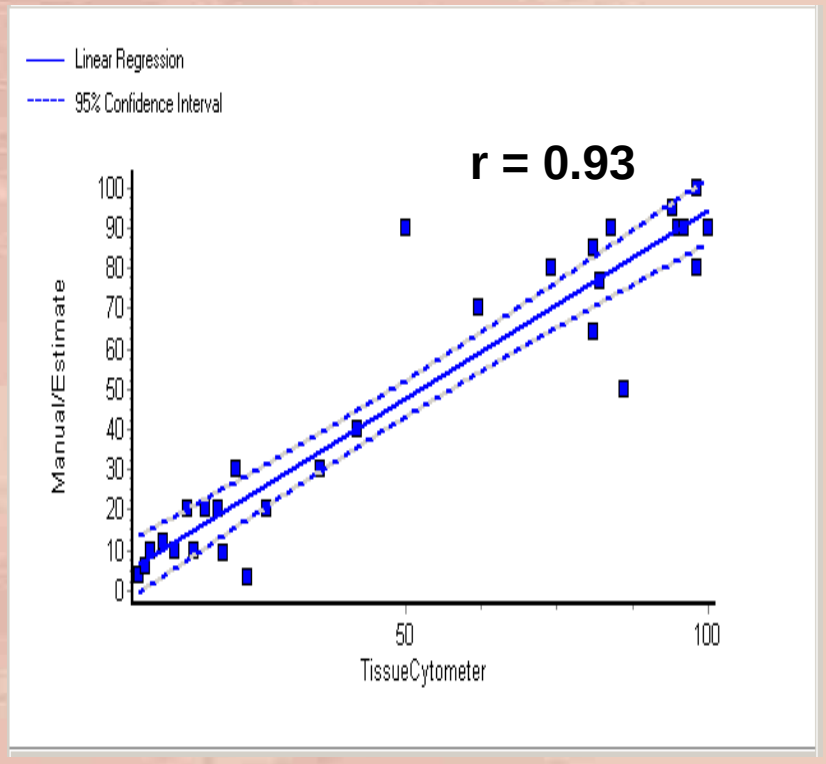
Tissue Cytometry (TC)-MCL Marker Profile Heterogeneity per patient

	S04-a	S04-B	S04 C	S04-D	**S04-D**	S04-E	S04-F	S02-G
BCL-1	94	81	79	87	82	50	62	81
CD3	36		31	58		27		
CD4	19		25	19	24	15		6
CD5	86		99		42	95	100	97
CD8	**14**	**20**	**7**	**12**	**7**	**8**	**6**	**5**
CD20	96	84	94	99	74	98	98	97
KI67	8	10	56	69	**81**	22		

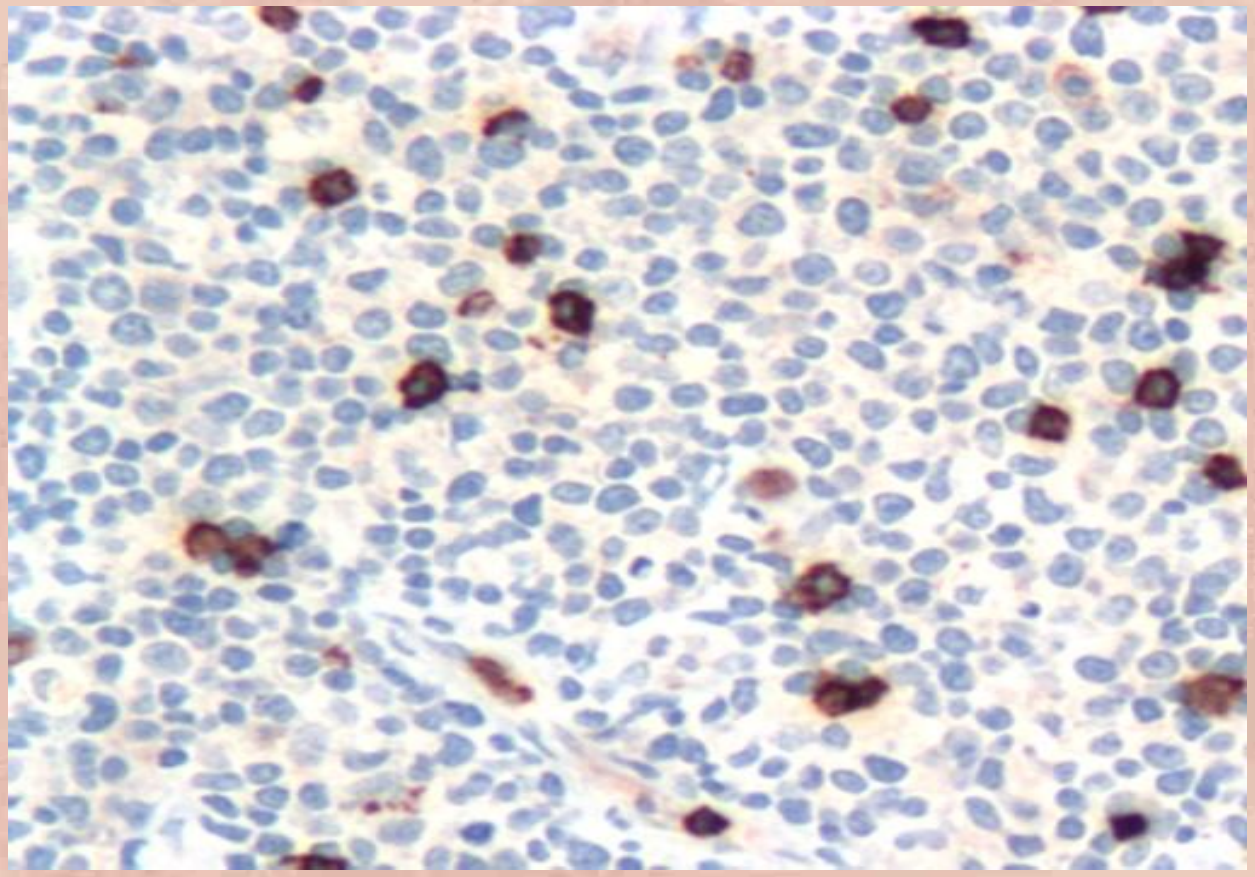
		S04-A	Y a		S04-c	y c
	TC	manual	flow	TC	manual	flow
bcl1	94	95		81	85	
cd3	36	30	8.4	24	25	29
cd4	19	20	6	1.9	20	21
cd5	86	50	89	80	85	75
cd8	14	20	5	5.8	9	8
cd20	96	90	86	84	90	66
ki67	8	10		10	12	

Correlation

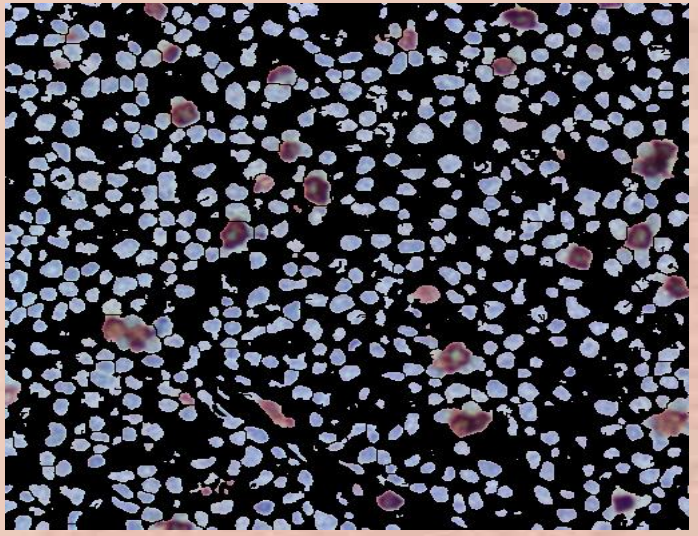


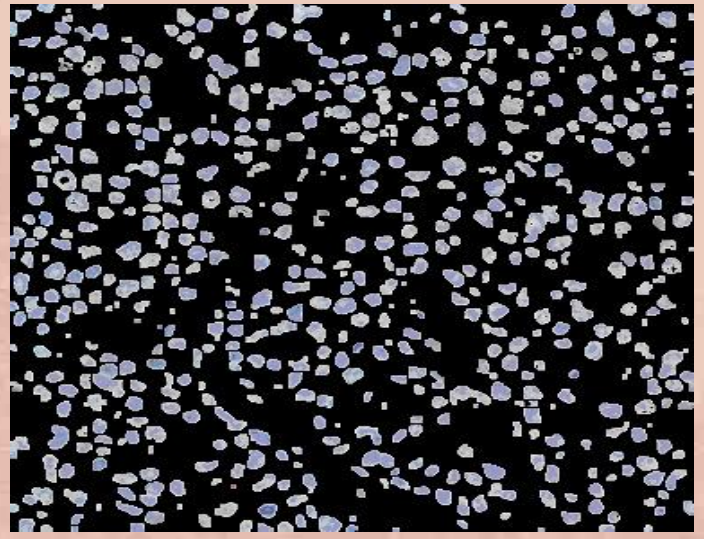


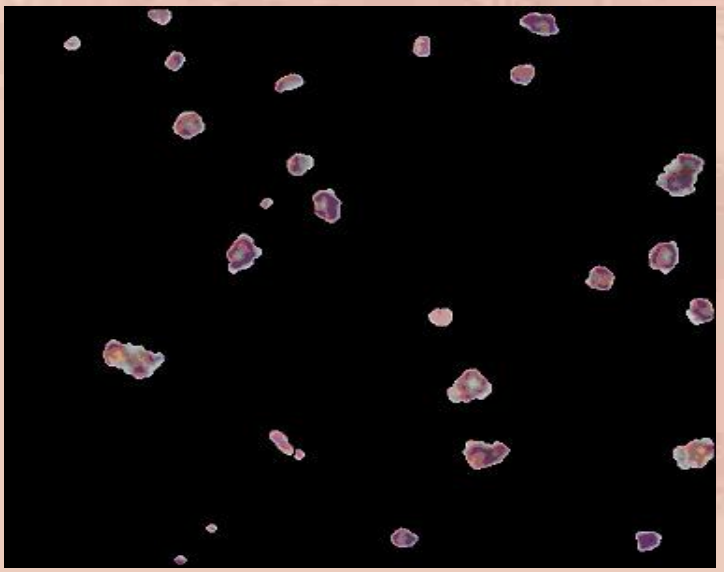
Virtual Flow cytometry



Segmentation and Result







#	Leucocyte_den	Lymphocyte_num	Lymph_stain_num	Lymph_stain_ratio(%)
1	4	511	26	5.088063
Min	4.00	511	26	5.088063
Max	4.00	511	26	5.088063
Median	4.00	511	26	5.088063
Total		511	26	
Average	2.56	511.000000	26.000000	5.088063

The results have been saved in the user1:case1:lymphres.dat

Cell based morphometric features all 511 cells

Label	%Area	Area	Mean	StdDev	Min	Max	X	Y	Perim.
1	cd8e8watershed	87	0	0	0	0	29	3	37
2	cd8e8watershed	216	0	0	0	0	113	7	59
3	cd8e8watershed	184	0	0	0	0	225	5	69
4	cd8e8watershed	66	0	0	0	0	336	4	35
5	cd8e8watershed	611	0	0	0	0	386	16	129
6	cd8e8watershed	103	0	0	0	0	419	6	39
7	cd8e8watershed	85	0	0	0	0	453	2	43
8	cd8e8watershed	137	0	0	0	0	471	5	47
9	cd8e8watershed	77	0	0	0	0	46	9	51
10	cd8e8watershed	123	0	0	0	0	159	7	41
11	cd8e8watershed	74	0	0	0	0	129	8	33
12	cd8e8watershed	122	0	0	0	0	11	10	41
13	cd8e8watershed	107	0	0	0	0	176	12	45
14	cd8e8watershed	76	0	0	0	0	216	12	35
15	cd8e8watershed	247	0	0	0	0	446	18	64
16	cd8e8watershed	185	0	0	0	0	492	13	50
17	cd8e8watershed	81	0	0	0	0	135	15	35
18	cd8e8watershed	128	0	0	0	0	147	19	42
19	cd8e8watershed	138	0	0	0	0	337	20	44
20	cd8e8watershed	109	0	0	0	0	4	23	42
21	cd8e8watershed	130	0	0	0	0	41	21	43

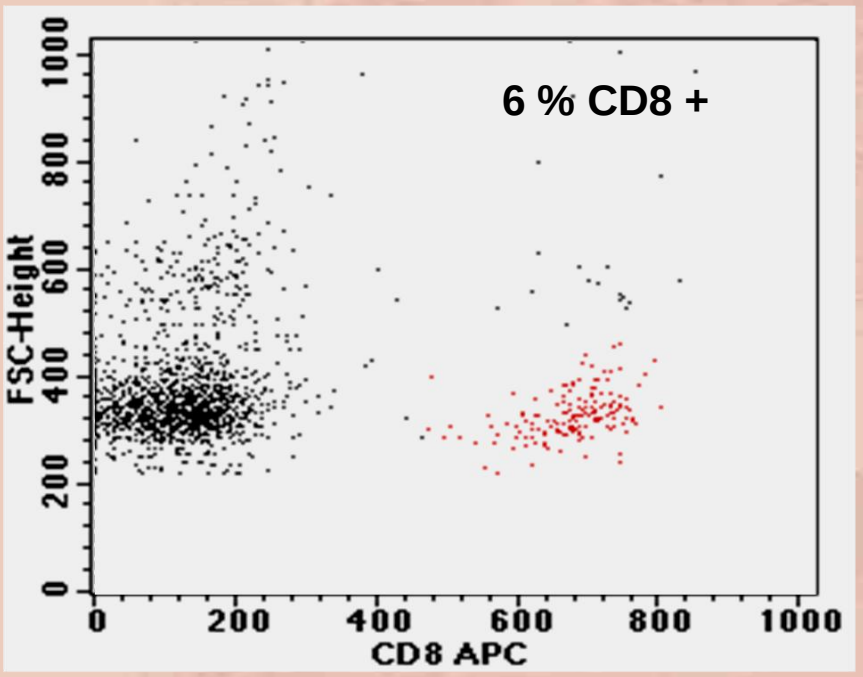
Cell based results on brown stained cells

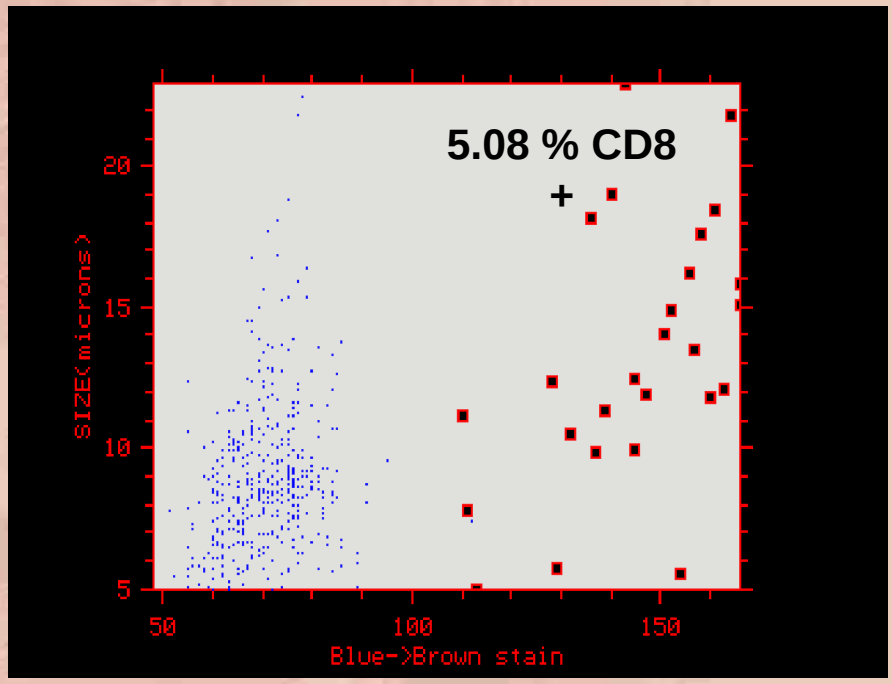
	Label	Area	Mean	StdDev	Min	Max	X	Y	F
	%Area								
1	21_watershednum 0	102	0	0	0	0	84	6	3
2	21_watershednum 0	230	0	0	0	0	287	11	5
3	21_watershednum 0	62	0	0	0	0	37	24	2
4	21_watershednum 0	106	0	0	0	0	224	26	3
5	21_watershednum 0	101	0	0	0	0	92	35	3
6	21_watershednum 0	142	0	0	0	0	278	43	4
7	21_watershednum 0	130	0	0	0	0	153	48	4
8	21_watershednum 0	227	0	0	0	0	99	75	5
9	21_watershednum 0	163	0	0	0	0	158	100	4
10	21_watershednum 4	476	11	52	0	255	362	106	8
11	21_watershednum 0	255	0	0	0	0	173	126	6
12	21_watershednum 0	35	0	0	0	0	141	124	2

cont

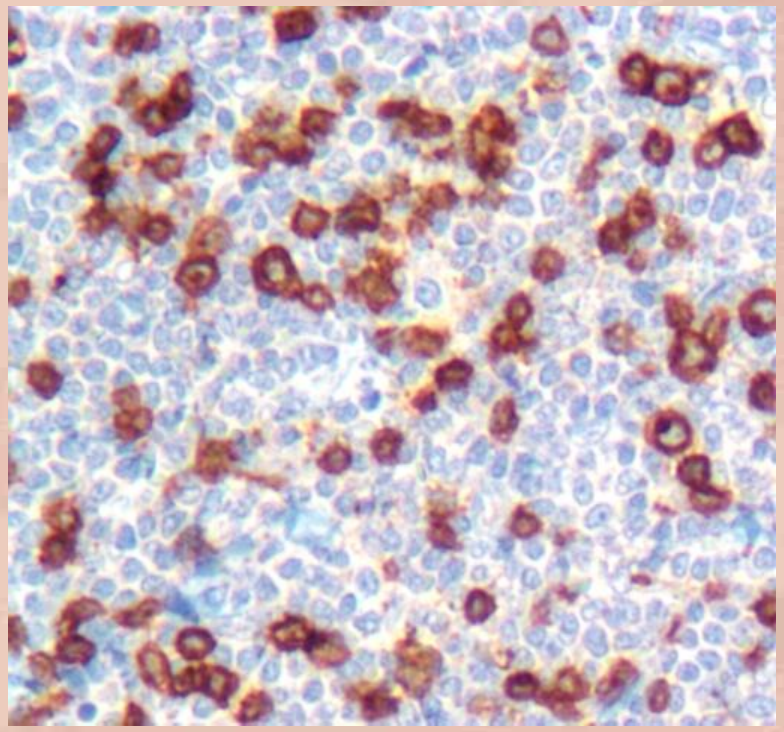
13	21_watershednum0_0	3100	0	0	0	0	128	156
14	21_watershednum0_0	2580	0	0	0	0	353	158
15	21_watershednum0_0	1820	0	0	0	0	319	172
16	21_watershednum0_0	1980	0	0	0	0	373	192
17	21_watershednum0_0	1260	0	0	0	0	234	196
18	21_watershednum0_0	5210	0	0	0	0	69	222
19	21_watershednum0_0	3610	0	0	0	0	249	240
20	21_watershednum0_0	1240	0	0	0	0	149	275
21	21_watershednum0_0	3330	0	0	0	0	370	282
22	21_watershednum8_63	3381	4	31	0	255	257	282
23	21_watershednum0_0	310	0	0	0	0	158	283
24	21_watershednum0_0	250	0	0	0	0	111	329
25	21_watershednum0_0	1380	0	0	0	0	214	335
26	21_watershednum0	145	0	0	0	0	345	337

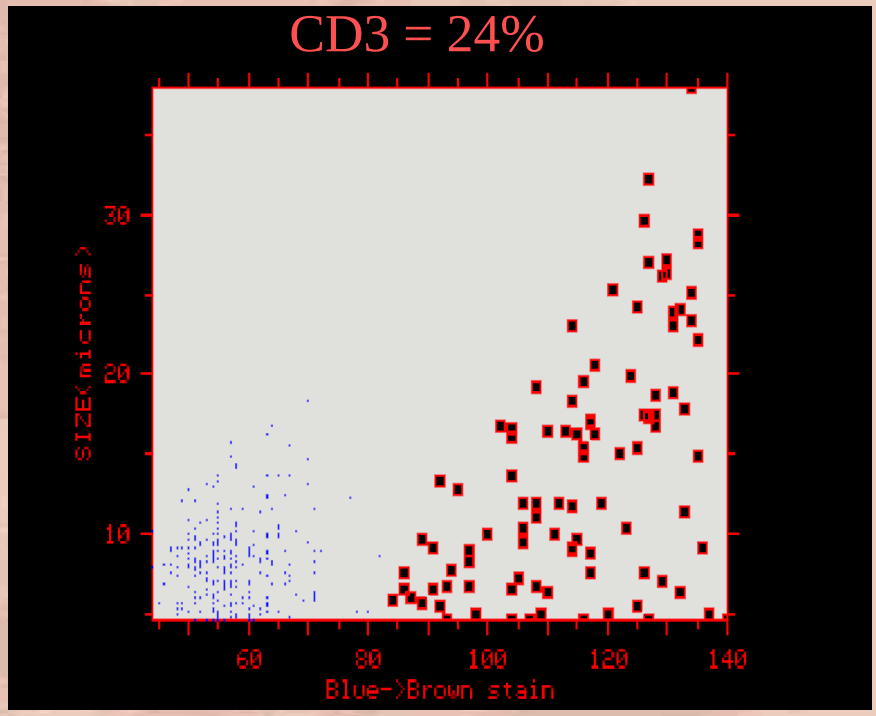
Results of Flow Cytometry and Virtual Flow Cytometry



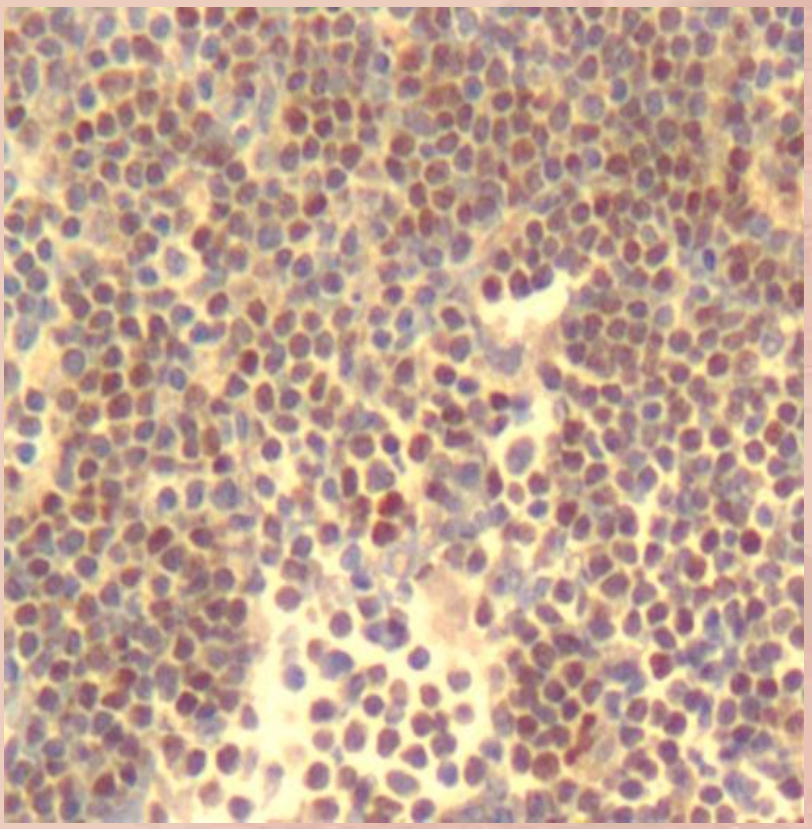


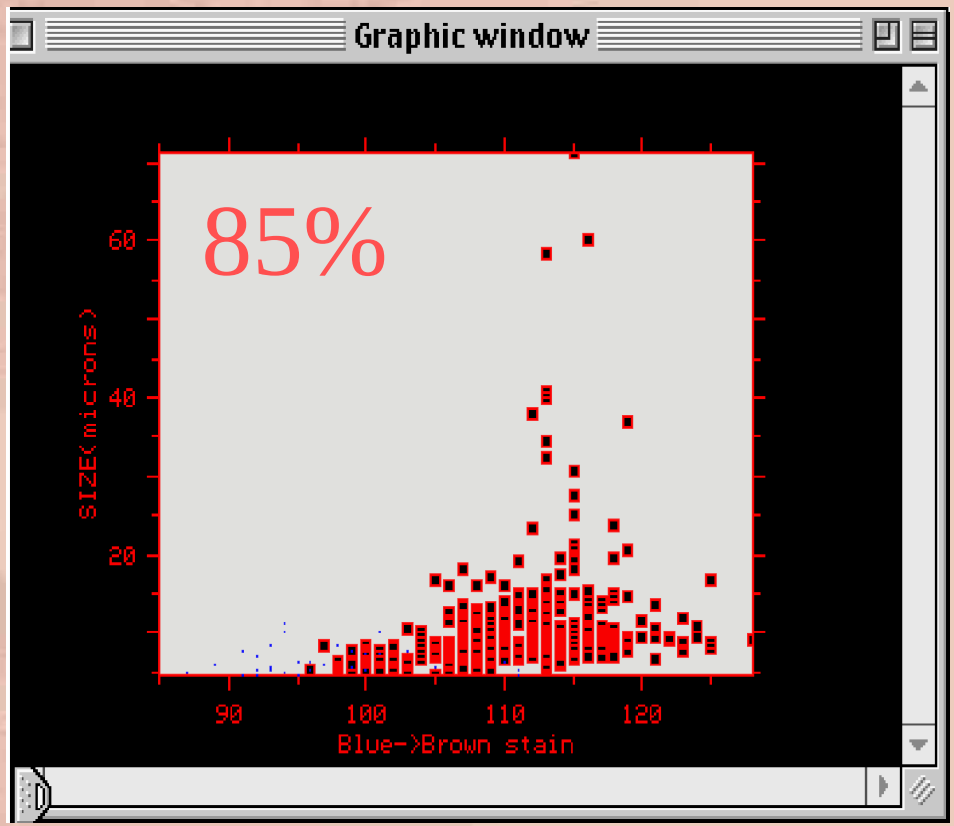
CD3



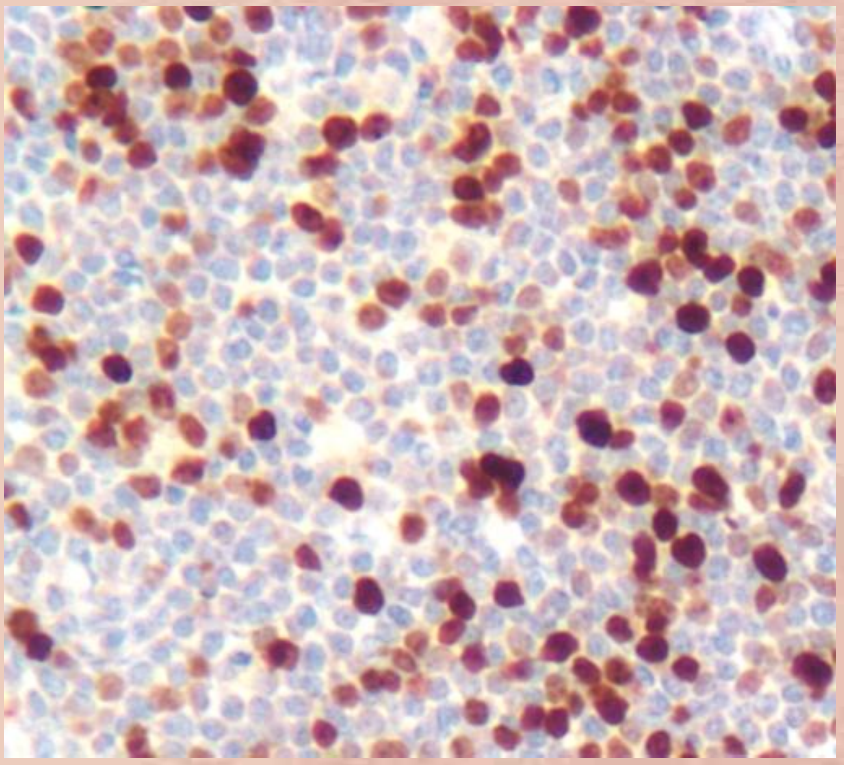


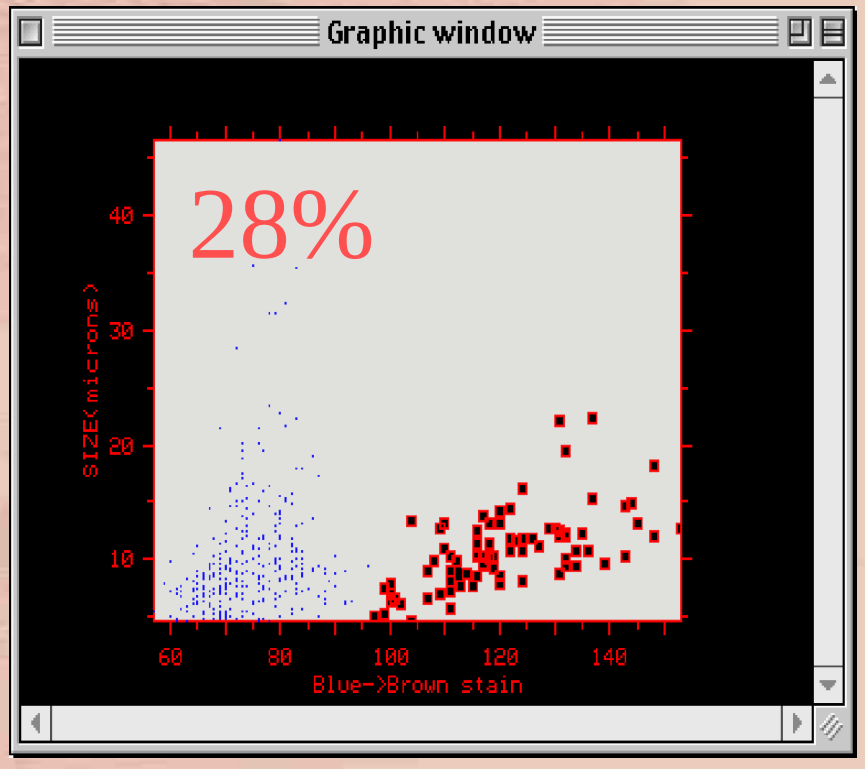
BCL-1




Graphic window
85%
SIZE(microns)
60
40
20
90
100
110
120
Blue->Brown stain

Ki-67




Graphic window
28%
SIZE(microns)
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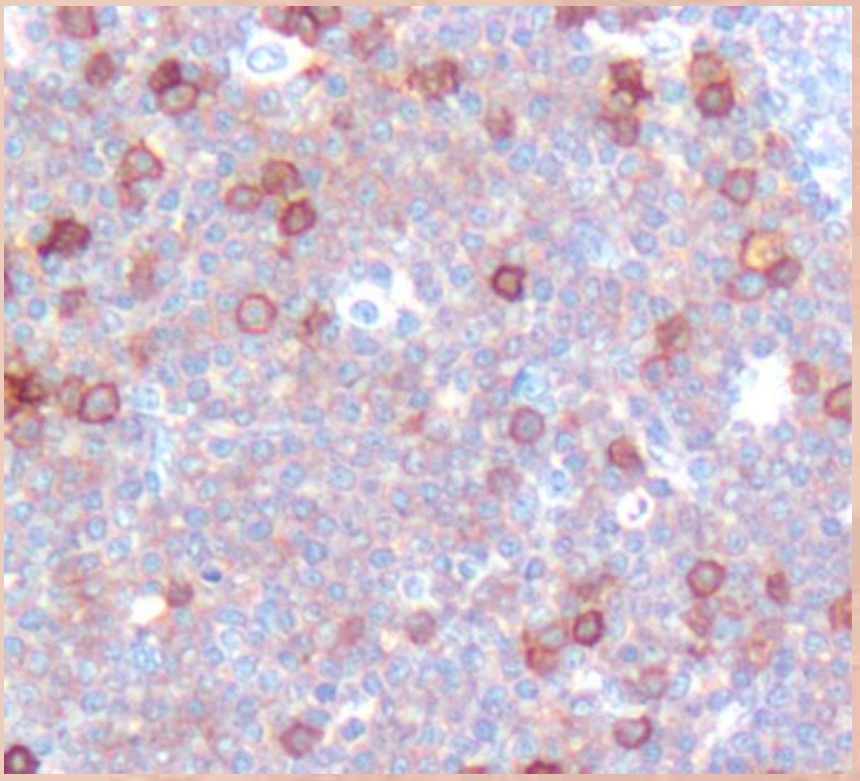
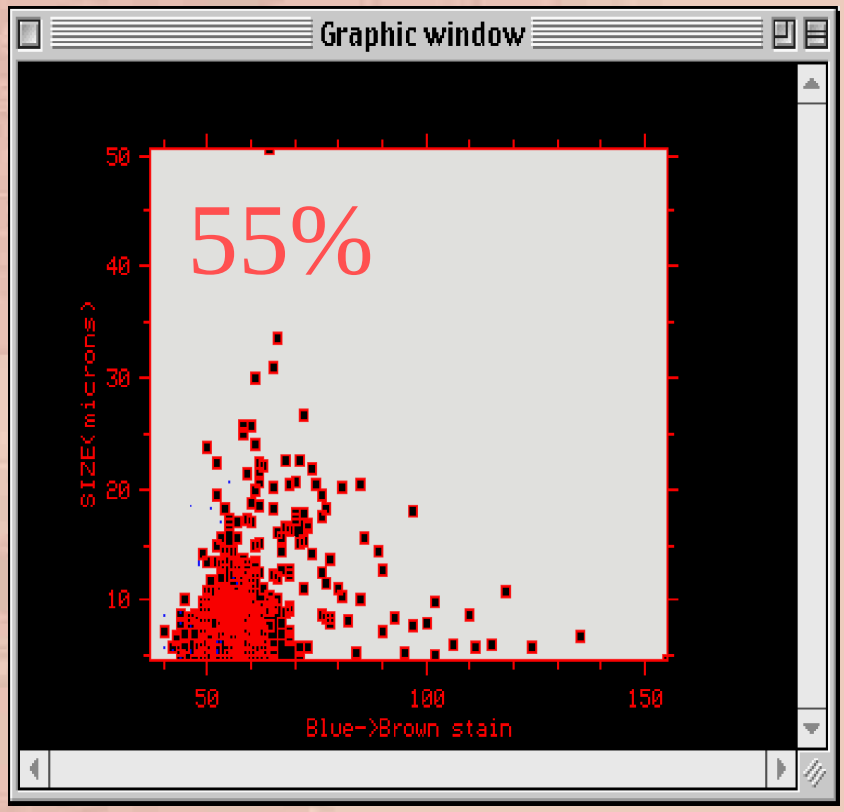
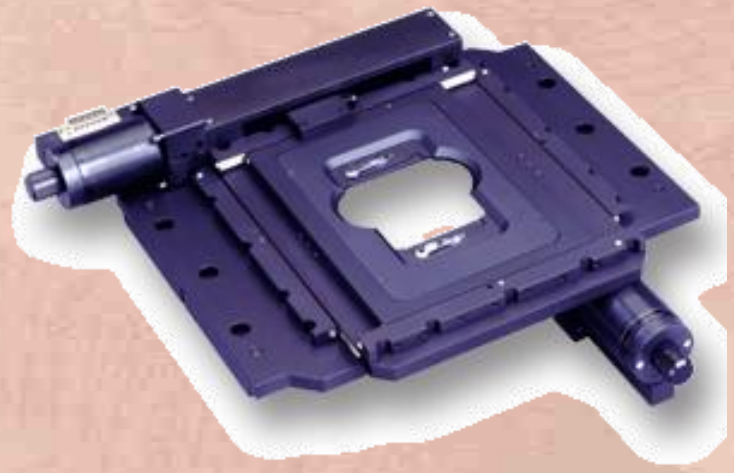
30
20
10
60
80
100
120
140
Blue->Brown stain

CD5




Graphic window
55%
SIZE(microns)
50
40
30
20
10
50
100
150
Blue->Brown stain

Additional automated capability: automated image identification and extraction- algorithm programmed

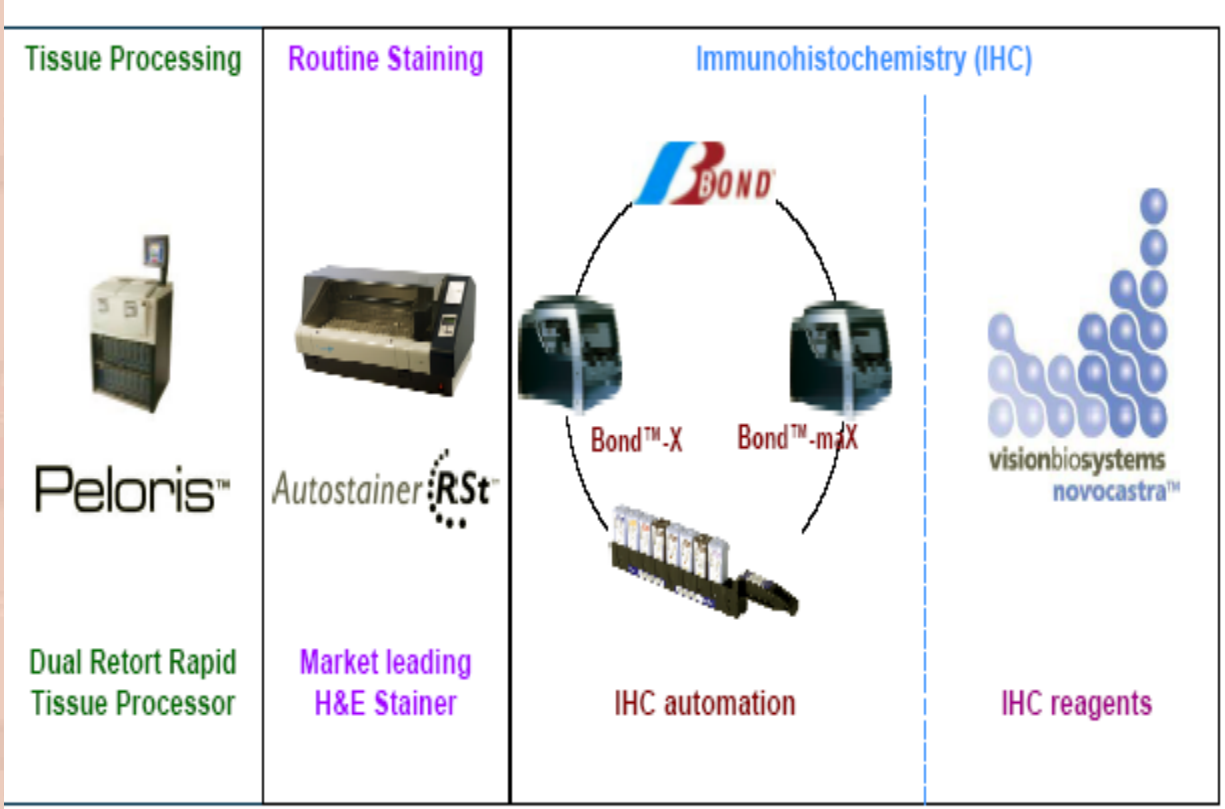


Ludl 3 x2 Biopoint X-Y stage and Z motor

Autofocusing algorithm for automated image extraction from slides

linked to MAC 2000 programming interface

Automated image analysis is the logical, unique, value added, super-extension of automated histology and immunohistochemistry



Automated

"ihcflow"

PLUS your slideloader with our software is the "Handle"

SL50 Automated Slide Loader

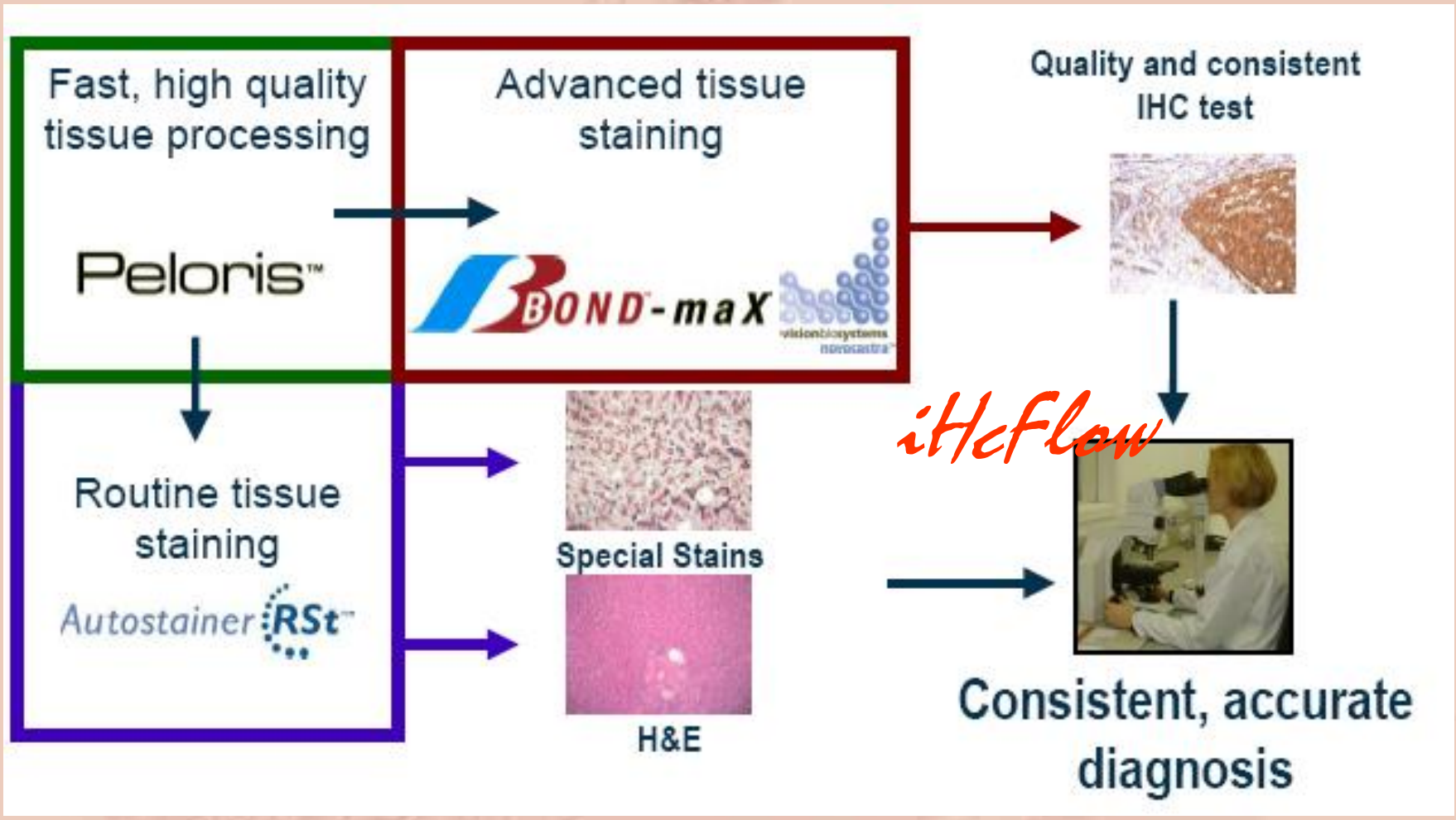
and Automated "*ihcflow*"

ENDPOINT application: THE "BLADE"-

Virtual Flow Report or IHCFLOWED slide – pathologist bills with global 88361 or 88180

-technical component $ 50

-26 prof component $ 100


Fast, high quality tissue processing
Peloris™
Advanced tissue staining
BOND-maX
Routine tissue staining
Autostainer RSt™
Special Stains
H&E
Quality and consistent IHC test
iHcFlow
Consistent, accurate diagnosis

Conclusion

Stain density, color and size information utilized by an advanced image analysis can perform **IHC marker profile cancer cytomics**.

We differ from the current methods of image analysis systems by focusing on **cell-based population statistics** instead of pixel-area data.

The correlation between each case run in flow cytometry and estimated by experts and by the advanced image Tissue Cytometry is high and suggest a **valid approach** to objectively quantifying immunostaining of lymph nodes in tissue by a defined set of monoclonal antibodies useful in lymphoma diagnosis, monitoring and prognosis.

This paradigm shift is a new **revenue platform** beyond the ER PR paradigm.

REFERENCES

Cualing H. Automated Analysis in Flow Cytometry. *Cytometry*, 2000 , 42:p.110-113.

Young IT, Quantitative Microscopy. *IEEE Engineering in Medicine and Biology*, 1996, 15(1): p.59-66.

Ridler TW ,Calvard S. Picture Thresholding Using Iterative Selection Method. *IEEE Trans. On Systems, Man, and Cybernetics*, 1978. SMC-8(8):p 630-632.

Cualing H. Kothari R, Balachander T. Immunophenotypic Diagnosis of Acute Leukemia Using Decision Tree Induction. *Lab Investigation*, 1999, 79:p.205-212.

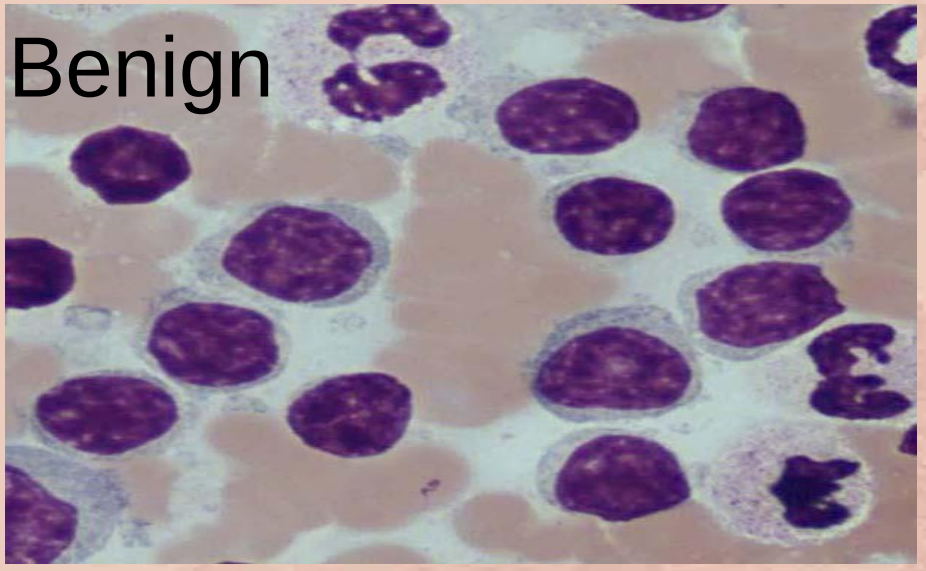
computerized tools in image cytometry

Slide based cytometry promises to be a tool to complement flow cytometry and enhance objectivity in histologic analysis

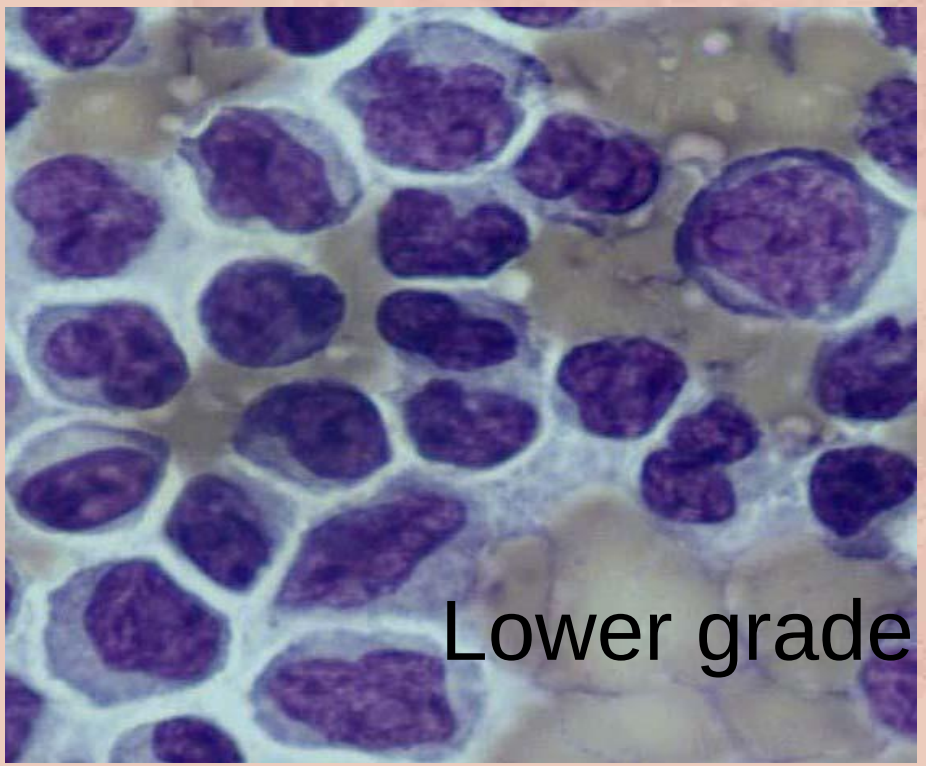
Niche:

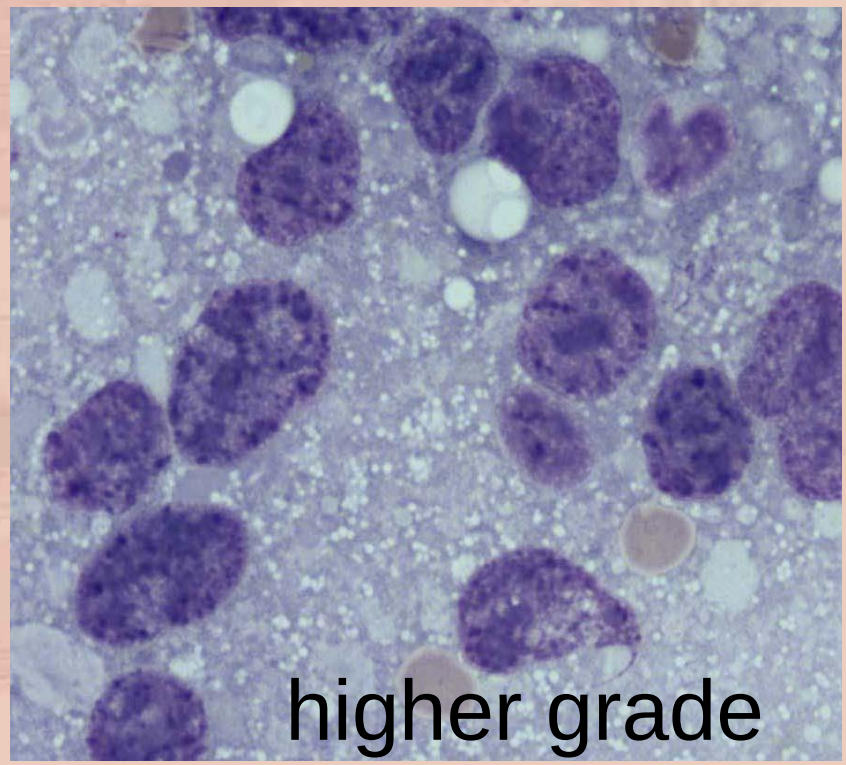
1.For tissue and cell-based quantitative analysis of immunohistochemistry stained cells

2. Logical extension of automation in histology and immunohistochemistry- iHcflow

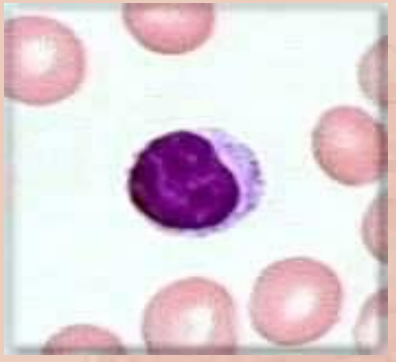


Routine stained imprints of Benign and malignant lymphocytes





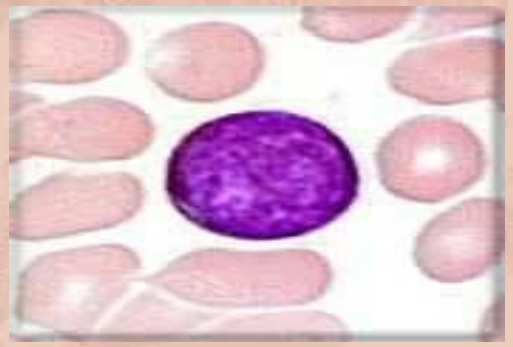
How do we then, measure the nuclear chromatin changes, that you and I see, in an objective quantifiable manner? ?



The chromatin textural changes we see as discriminants could be seen in both benign and malignant lymphocytes

Atypical lymphs

Burkitt's cell



- Lymphoblast

Clumped vs dispersed

Data used

- **51 benign cytologic imprints**
- **164 malignant lymphoid cytologic Romanowsky imprints of low grade, intermediate, high morphologic grade of NHL lymphoma are digitized**
- **The digitized gray scale images are analysed through a cell recognition system- 1.segmentation, 2. Feature extraction, 3. Classification**

What does the image cytometer sees?

Course nuclear textures-larger changes of pixel gray happen with distance- large variance of pixel density

Fine nuclear texture-changes of pixel gray happen mildly with distance-small variance

-

2. Feature Extraction

1. Variance (measure of contrasts)
2. Mean (measure of pixel brightness)
3. Skewness (measure of assymetry)
4. Kurtosis (measure of peakedness)
5. Entropy (high when even texture)
6. Elemental order (low with varied texture)
7. Uniformity (high with course texture)
8. Homogeneity (high with even texture)
9. Deviation from circularity (ellipse or round)
10. Curvature (irregularity of contours)

RESULTS: 10 Dimensions/Features per nucleus; dimensions further combined and mathematically expanded

3. Classification-based on similarities in multi-dimensional feature space.

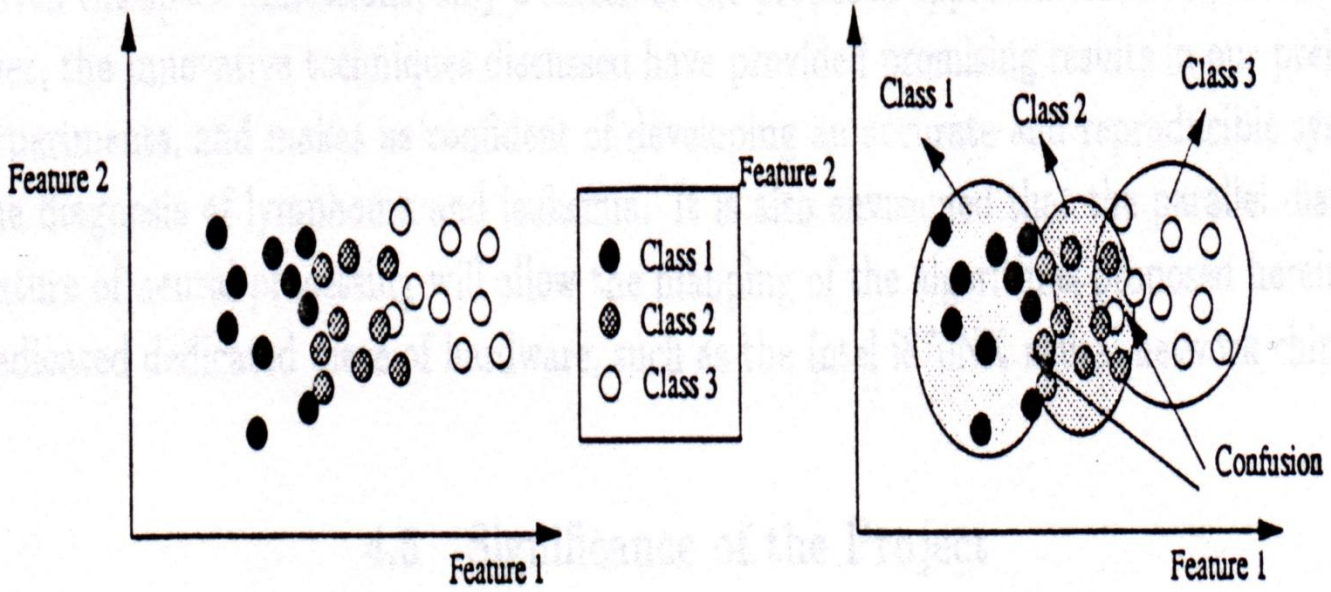
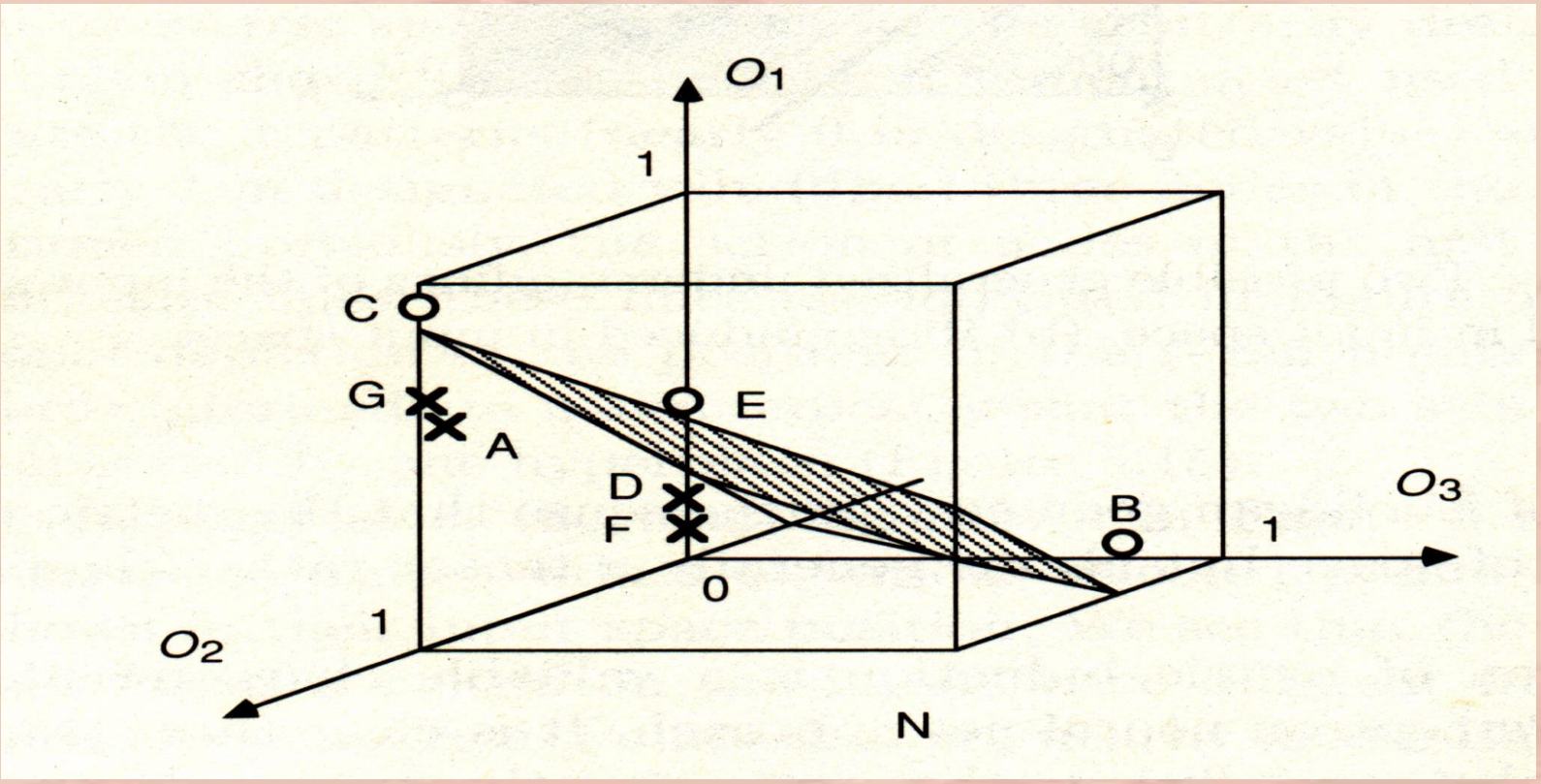


Figure 5: From left to right. (a) Overlapping features in the feature space, (b) Response of a non-attentional classifier. Note the confusion in discriminating between Class 2 and Class 1, and Class 3 and Class 2

k-NN classifier- based on classification of similarity of features analysed on more than 3 feature-dimensions is used.



Example of visualization of 3 dimensional feature space and the created 3-d hyperplanes boundary.

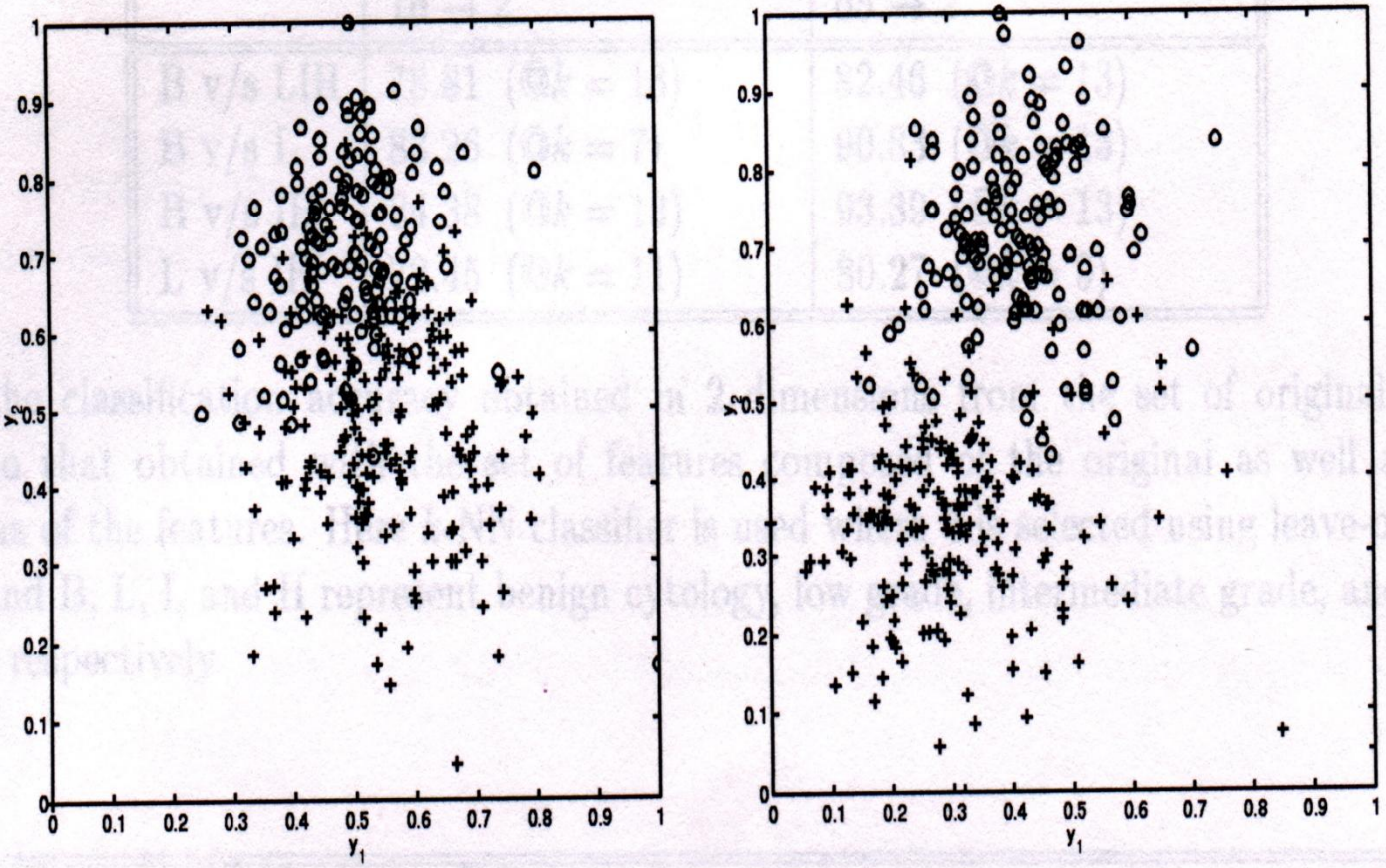
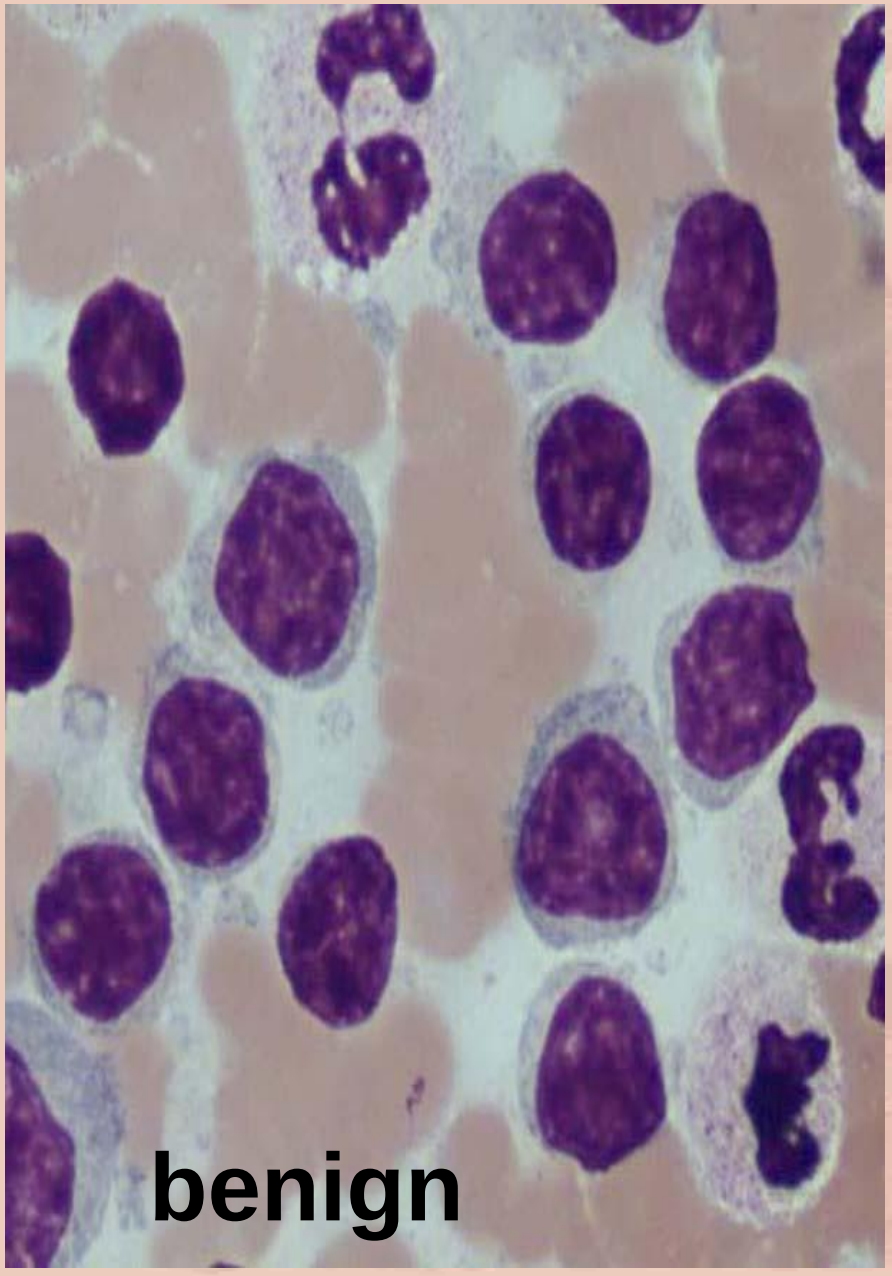
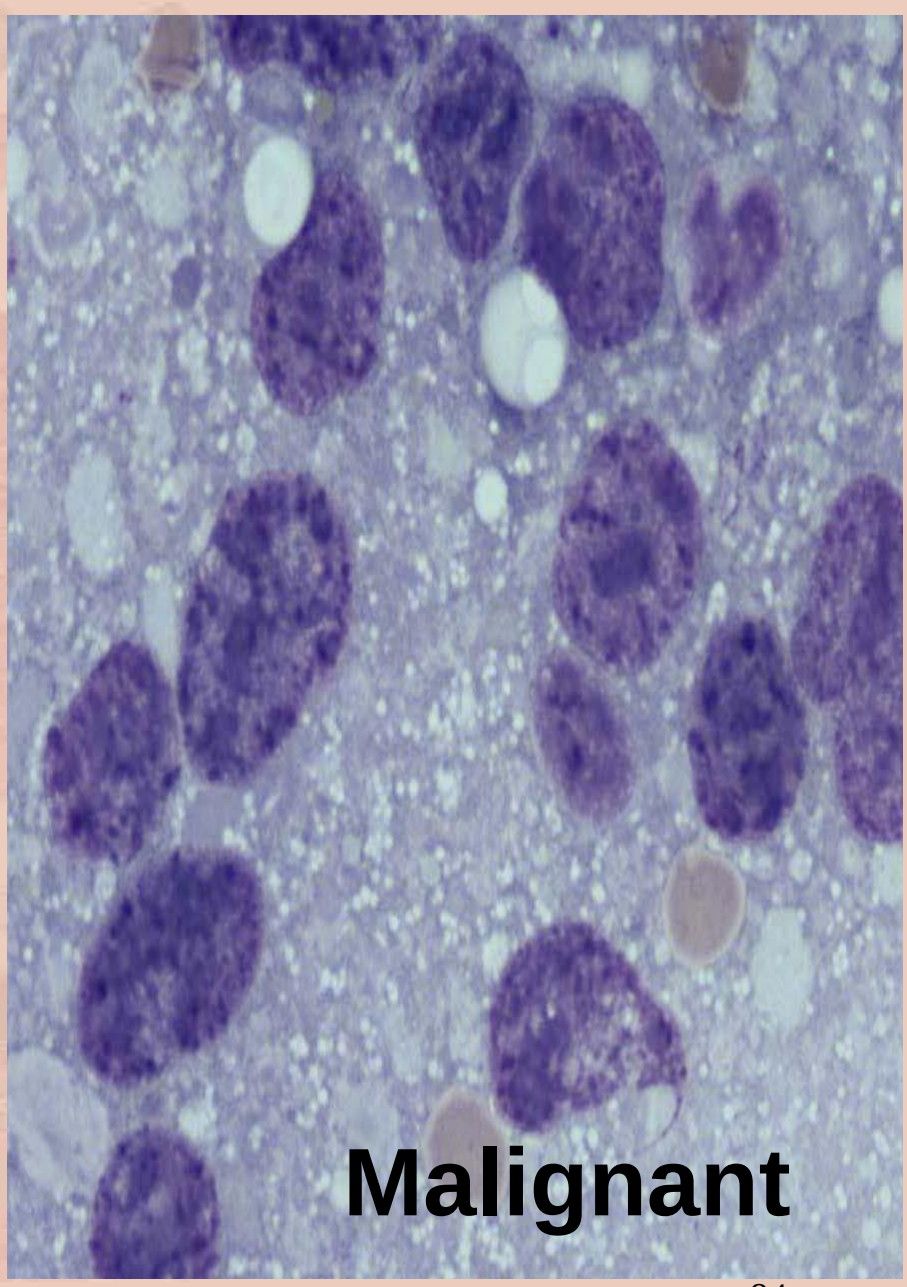


Figure 6: Separability of benign cytology (represented by a o) from intermediate and high grade lymphomas (represented by a +). The left panel shows the separability in 2 dimensions from the original features, while the right panel shows the separability in 2 dimensions of the data comprising the original features and distinct quadratic combinations of the original features


benign


Malignant

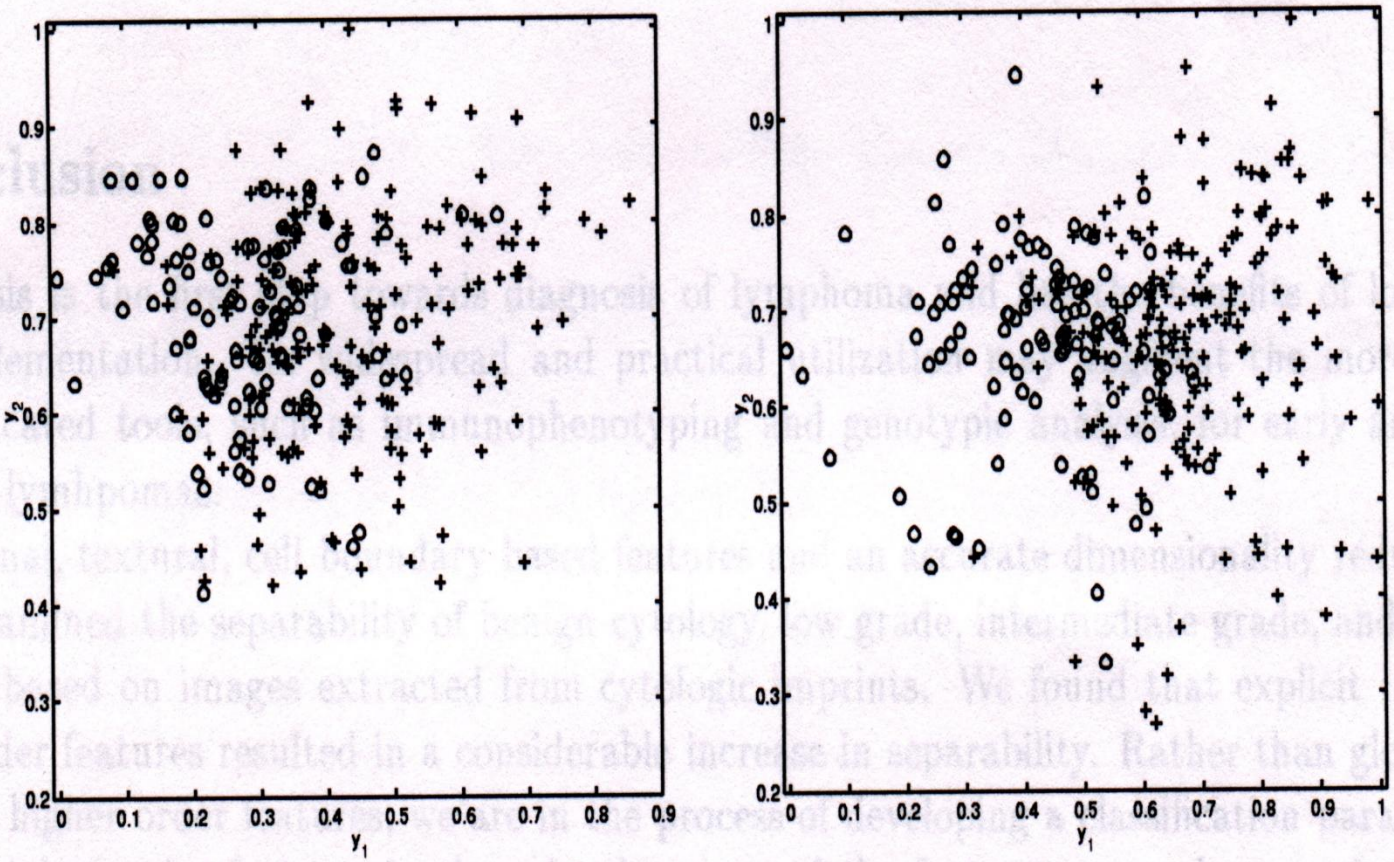
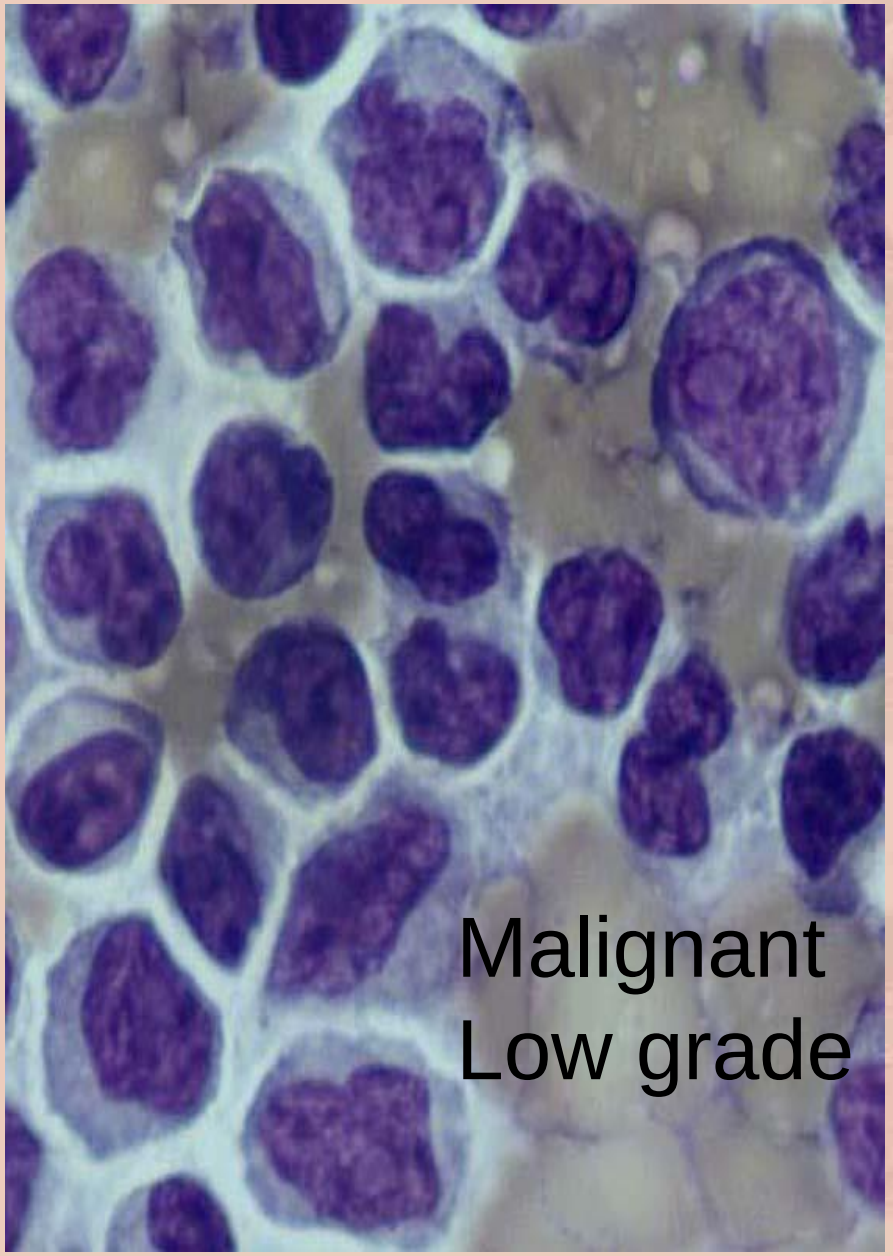
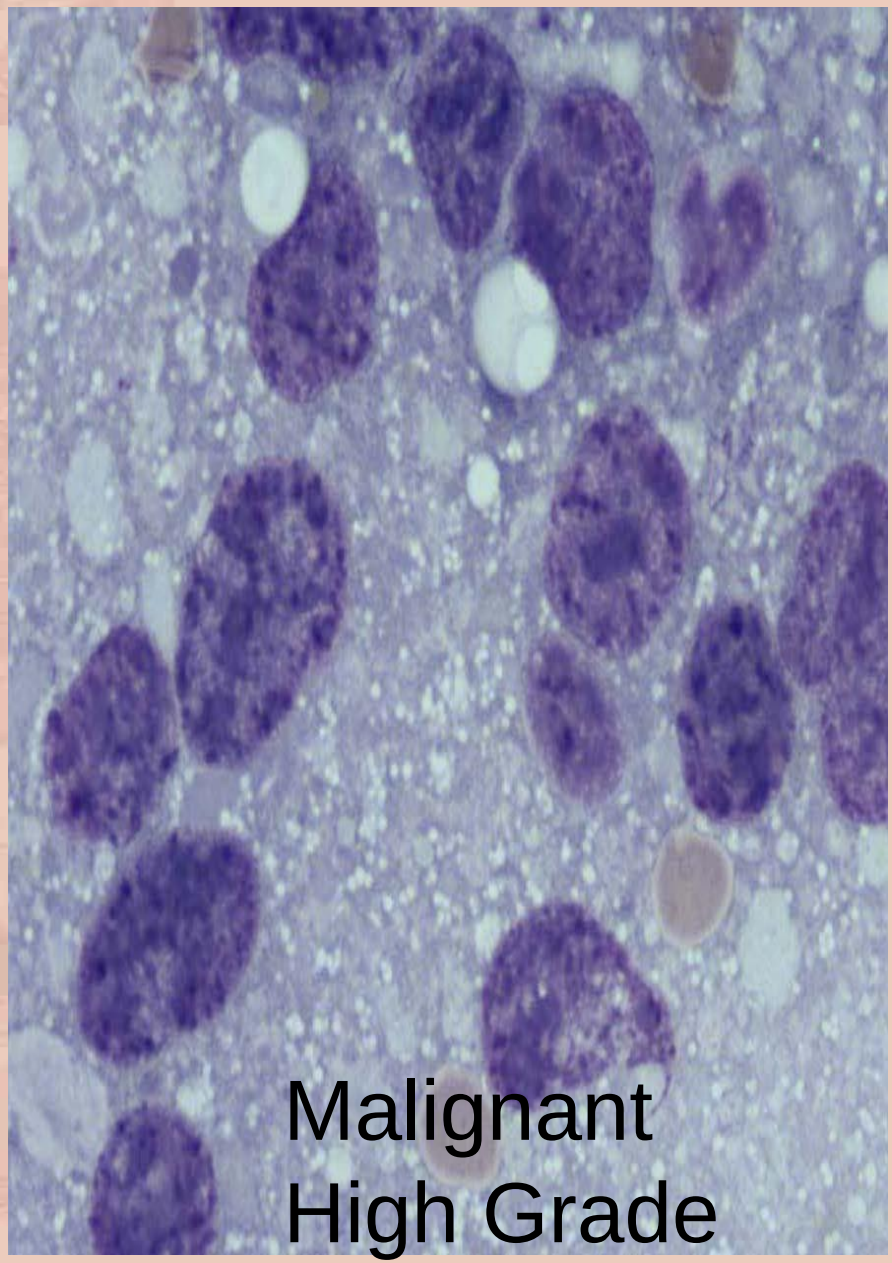


Figure 7: Separability of low grade (represented by a o) from combined intermediate and high grade lymphomas (represented by a +). The left panel shows the separability in 2 dimensions from the original features, while the right panel shows the separability in 2 dimensions of the data comprising the original features and distinct quadratic combinations of the original features


Malignant
Low grade


Malignant
High Grade

	Classification accuracy $10 \rightarrow 2$	Classification accuracy $65 \rightarrow 2$
B v/s LIH	78.81 $(@k = 13)$	82.46 $(@k = 13)$
B v/s L	83.26 $(@k = 7)$	90.83 $(@k = 13)$
B v/s IH	84.38 $(@k = 13)$	93.39 $(@k = 13)$
L v/s IH	72.45 $(@k = 11)$	80.27 $(@k = 9)$

Table 1: The classification accuracy obtained in 2 dimensions from the set of original features as compared to that obtained with the set of features composed of the original as well as quadratic combinations of the features. Here k-NN classifier is used where k is selected using leave-one-out cross validation and B, L, I, and H represent benign cytology, low grade, intermediate grade, and high grade lymphomas respectively

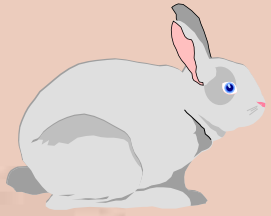
Interpretation:

The nuclear features that most separate benign from malignant are:

1.High variations and contrasts of gray levels in benign, less variations in malignant
2. Peaked chromocenters more in benign
3. Repetitive chromatin pattern in benign than in malignant
4. Denser, darker chromatin more seen in benign
5. Deviation from circularity more in malignant
6. Larger shift of pixel patterns in benign

These descriptors now have dimensions, quantifiable and may be used by a cytometer

Coming up above ground



1) Mine the data results of image cytometry to include in an automated cytometer to be "extra set of eyes" to pathologists

2) come up to the surface with an idea for a tool: a *hue discriminating image cytometer* for everyday pathology diagnosis and biologic research application

Table 1. Panel of Lineage and Differentiation Markers Used*

Antibody	Source	Specificity
IgA	Kallestad	® B cell surface IgA
IgD	Kallestad	® B cell surface IgD
IgG	Kallestad	® B cell surface IgG
IgM	Kallestad	® B cell surface IgM
Lambda	Dako	® B cell surface light chain
Kappa	Dako	® B cell Kappa light chain
CytoMu	Coulter	® B cell cytoplasmic IgM
CD1a	Ortho (T6)	(N) Immature T, Langerhan cells
CD2	Coulter (T11)	(N) T, NK-cell
CD4	Coulter (T4)	(N) T subset, macrophage
CD5	BD (Leu-1)	(N) T subset, B subset
CD7	Coulter (3A1)	(N) Precursor T, T subset, NK
CD8	Coulter (T8)	(N) T subset, etc
CD10	BD (CALLA)	(N) Precursor B, granulocyte
CD11C	BD (LeuM5)	(N) Gran, macrophage, platelets
CD13	Coulter (MY7)	(N) Granulocytes, Macrophage
CD14	Dako (M02)	(N) Macrophag, Gran, Langerhans
CD15	BD (LeuMI)	(N) Gran, macrophage, RS cell
CD19	Coulter (B4)	® Precursor B, mature B cell
CD20	BD (Leu-16)	® Precursor B subset, mature B
CD24	Accurate (BA-1)	(N) B cells, granulocytes
CD33	Coulter (MY9)	® Precursor myeloid
CD34	AMAC (HPCA-1)	(N) Progenitor cell, etc
CD38	BD (Leu17)	(N) Progenitor, NK, T, plasma
CD41a	AMAC (PLT-1)	® Platelets
HLA-DR	BD (HLA-DR)	(N) B cell, activated T, myeloid
TDT	MGR (Terminal deoxynuc. trans.)	(N) Lymphoblastic T B, Myeloid

* ®, restricted; N, not restricted; RS, Reed-Sternberg; T, T cell; B, B cell; Ig, immunoglobulin totality of reactivity not all shown; Gran, granulocytes.

Table 4. Example Cases with Flow Cytometry Results* (%)

	ALL	R	MLL	AML	AML ◇ # MLL	R ◇ AML ##
CD2	6	77	5	5	12	41
CD20	86	1	1	5	4	0
CD19	86	4	93	2	29	9
CD4	6	1	9	0	0	0
CD8	3	1	2	0	0	0
IgG	0	2	7	0	0	0
IgM	0	1	3	0	0	0
IgA	0	2	3	0	0	0
IgD	0	1	5	0	0	0
KAPPA	2	3	5	0	0	24
LAMBDA	2	2	2	0	0	20
HLADR	57	0	96	57	81	32
CALLA	98	0	96	1	2	6
TdT	99	0	82	6	42	0
CytMU	0	0	30	0	0	0
CD24	4	0	82	0	0	0
CD38	1	0	20	0	0	0
CD13	1	8	69	86	69	0
CD14(MY4)	0	0	1	13	0	11
CD33	0	0	1	92	46	28
CD11c	0	0	1	33	0	0
CD14(Mo2)	0	0	20	0	0	0
CD15	0	0	20	90	0	0
CD1a	0	0	20	0	0	0
CD5	4	60	4	0	0	0
CD7	97	55	6	4	0	0
CD34	87	0	65	65	85	0

* ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; R, reactive marrow; MLL, mixed lineage leukemia, ◇, discrepant cases for which classifier disagreed (previous diagnosis ◇ ID3 diagnosis); #, 2 myeloid, 1 lymphoid satisfied EGIL criteria for ML; ##, morphologically remission hypocellular marrow with many maturing myeloid cells, has CD2 but lacks reactive CD5.

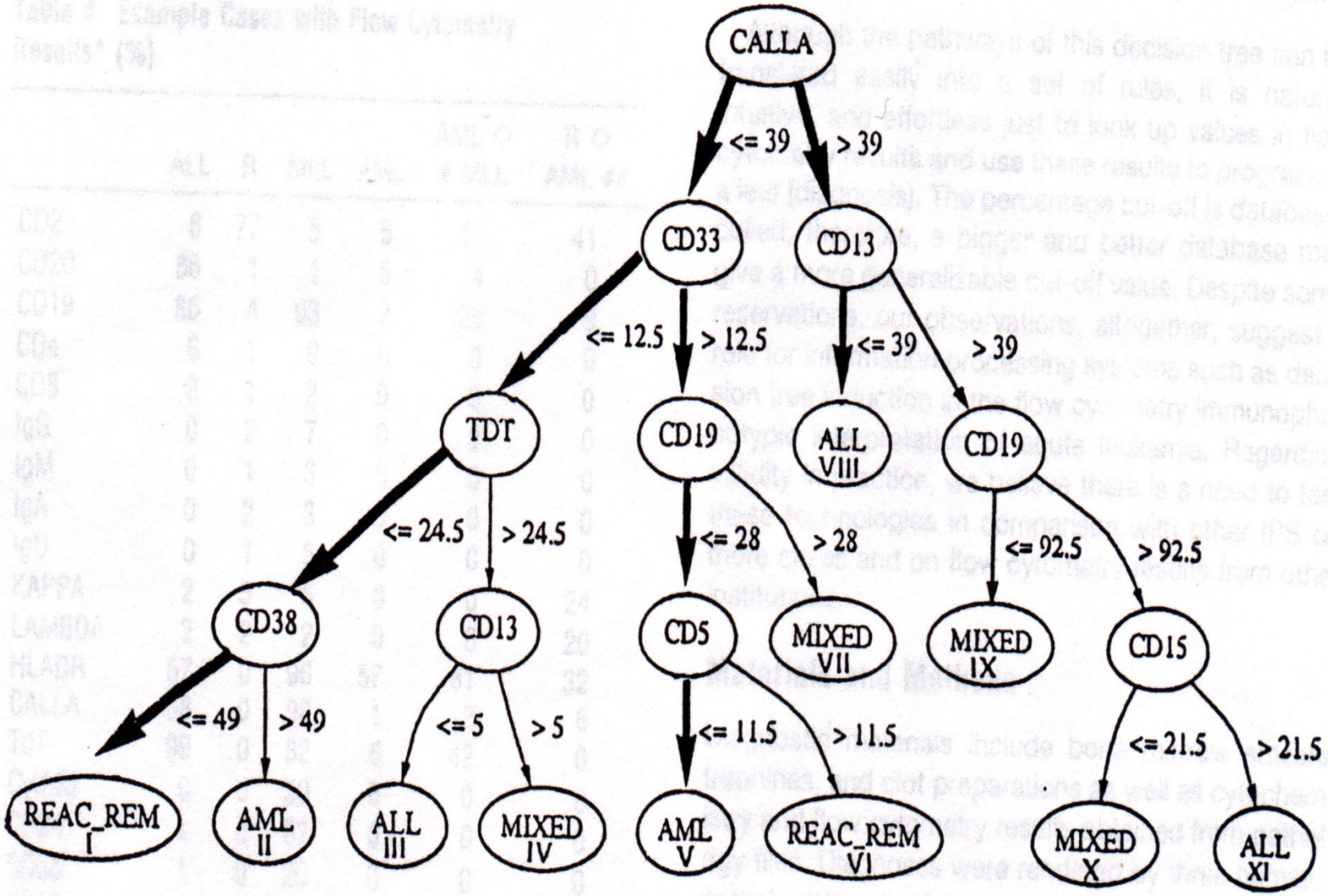


Figure 1.
Decision tree analysis.

Table 3. Frequency of Reactivity in Acute Lymphoid Leukemia (ALL), Reactive Marrow (RM), Mixed Lineage (MLL), and Acute Myeloid Leukemia (AML)

	Pos[a] (+)	ALL (%)	RM[b] (%)	MLL (%)	AML (%)
CD2	61	18	55	4.9	8.6
CD20	32	84	9.3	3.1	3.1
CD19	81	62.9	13.5	18.5	4.9
CD4	11	27	63	10	0
CD8	11	28	72	0	0
IgG	8	26	37	0	37
IgM	2	50	50	0	0
IgA	1	0	100	0	0
IgD	1	100	0	0	0
KAPPA	8	50	13	12	25
LAMBDA	0	0	0	0	0
HLADR	108	50	12	12	26
CALLA	78	86	4	7.5	2.5
TdT	101	77	0	12	11
CytMU	13	84	8	8	0
CD24	38	92	5	3	0
CD38	6	83	0	0	17
CD13	48	4	2	4	90
CD14(MY4)	3	0	0	50	50
CD33	57	5	9	14	72
CD11c	23	13	4	9	74
CD14(Mo2)	4	0	0	50	50
CD15	37	8	3	24	65
CD1a	2	100	0	0	0
CD5	30	43	57	0	0
CD7	69	36	38	2	24
CD34	61	70	0	10	20

[a] The blast gate was used and not the total-cell plot; ≥20% staining cells are called positive.

[b] 40% are in postchemotherapy remission.

Basic morphometry has role BM cellularity

- All BM cellularity are estimates