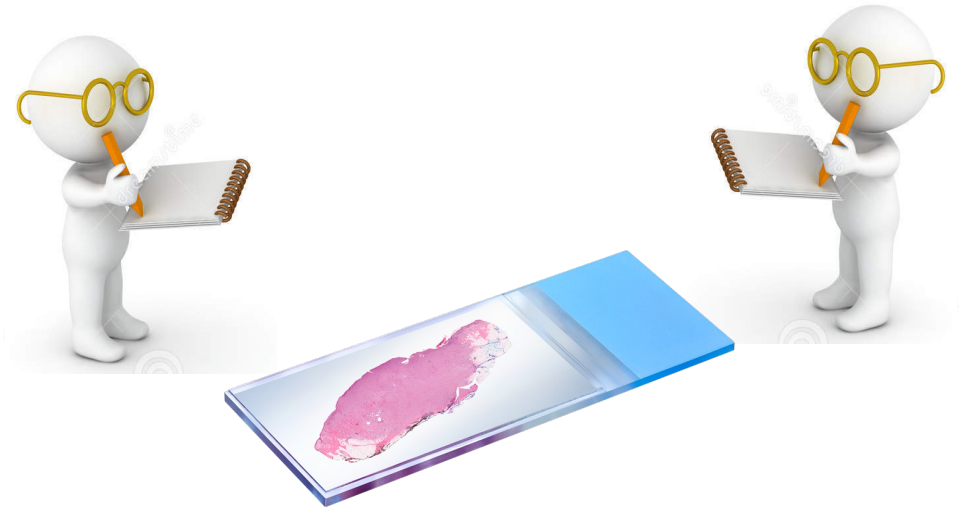
Cellular Pathology

Lecture #22

Advanced Histological Techniques:

Flow Cytometry in Pathology



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Lecture Outline

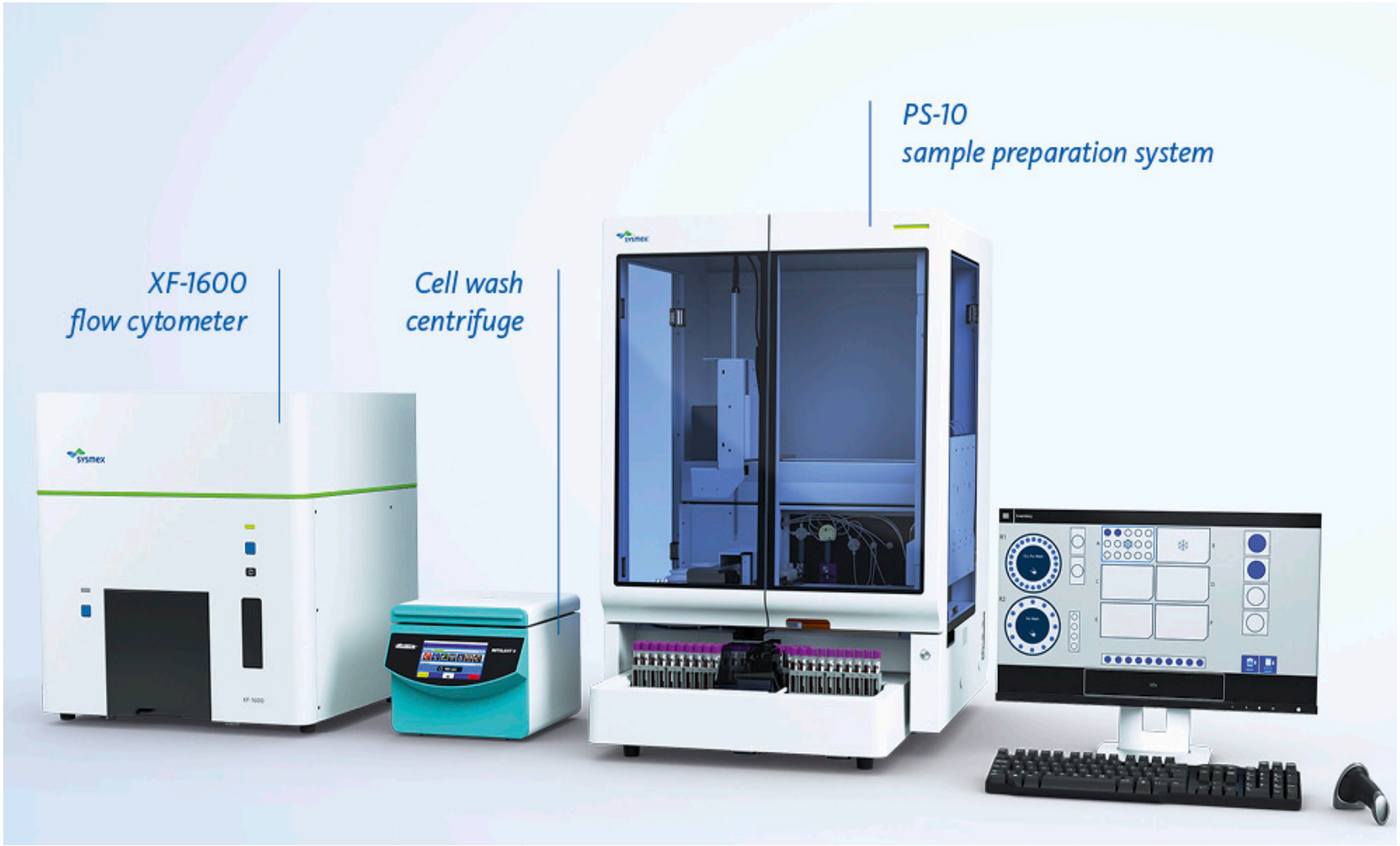
- Principle of Flow Cytometry
- Fluorochrome Dyes Used used in FCM
- Samples for Flow Cytometry
- Targets of Applications
- DNA Content & Ploidy Analysis
- Clinical Application
- Limitations of FCI

Learning Objectives:

At the end of this lecture, the student is expected to:

- Understand the principle of flow cytometry
- Know the clinical applications of flow cytometry in Pathology
- Appreciate the advantages and limitations of flow cytometry in Pathology
- Appreciate the Fluorochrome Dyes Used used in FCM
- Understand how flow cytometry is used in DNA Content & Ploidy Analysis

Flow Cytometry



Synopsis

- **Flow Cytometry** (FCM) is the method by which the various characteristics of individual particle or cells are studied.
- FCM provides us very quick assessment of cell surface antigens, DNA content and intracellular proteins.
- It is now a well-established technique for the diagnosis and classification of lymphoid neoplasms, identification of malignant cells in effusion cytology and other body fluids.

Principle of Flow Cytometry

- Single dissociated cells in liquid medium are essential for flow cytometry. The specific component of the cell is identified by the antibody tagged with a fluorescent dye.
- Similarly DNA can also be stained by a DNA stoichiometric dye.
- The single cells rapidly pass in front of a laser beam, and the laser beam of particular wave length hits the cell. The individual cells absorb the light and emit light of different wavelength.
- The emitted light is detected by the photomultiplier tube and is converted into a digital pulse. The intensity of the digital signal is stored in the computer and expressed in a relative scale known as channel. The result of the events is expressed as dot plot or histogram.

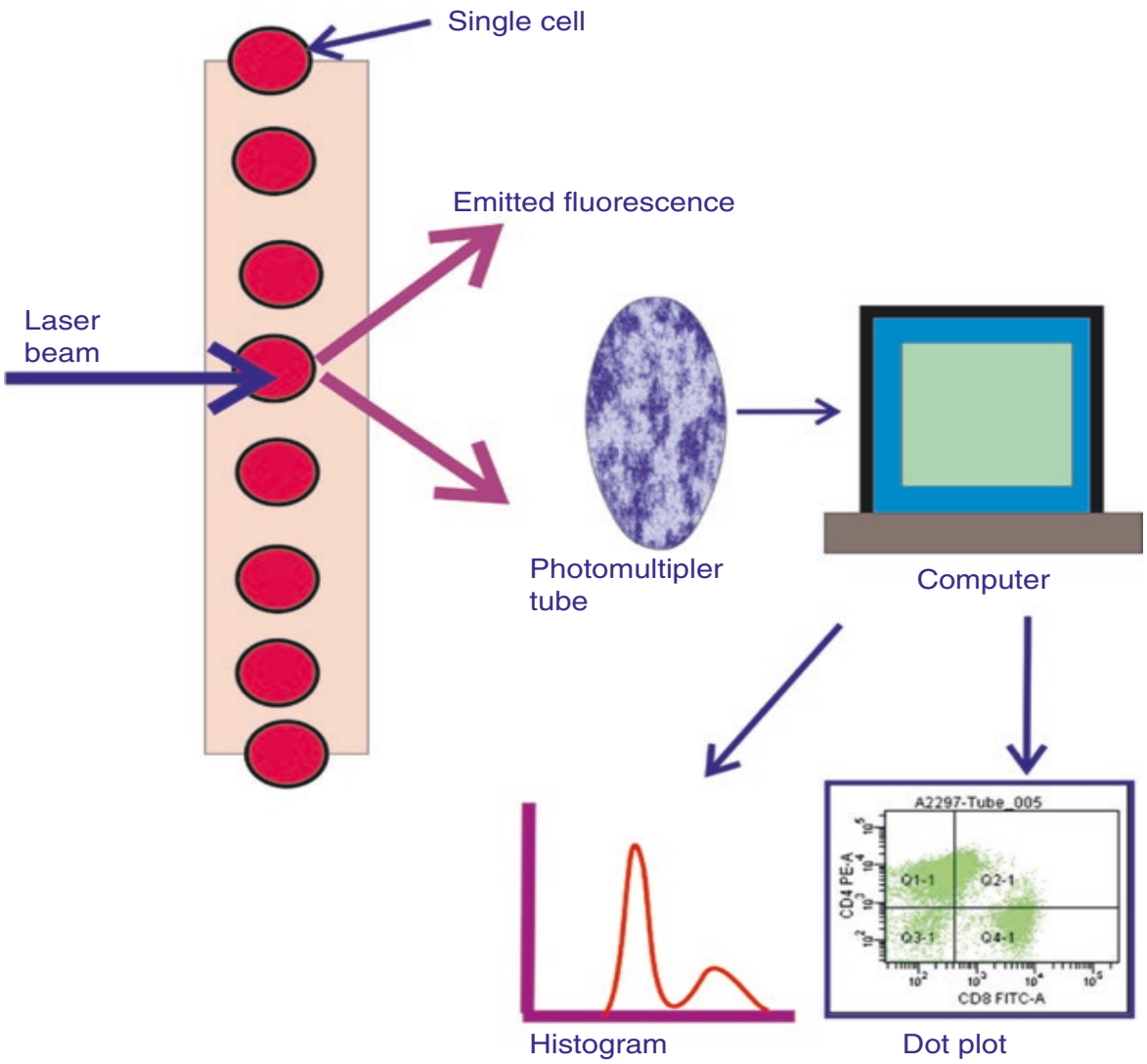
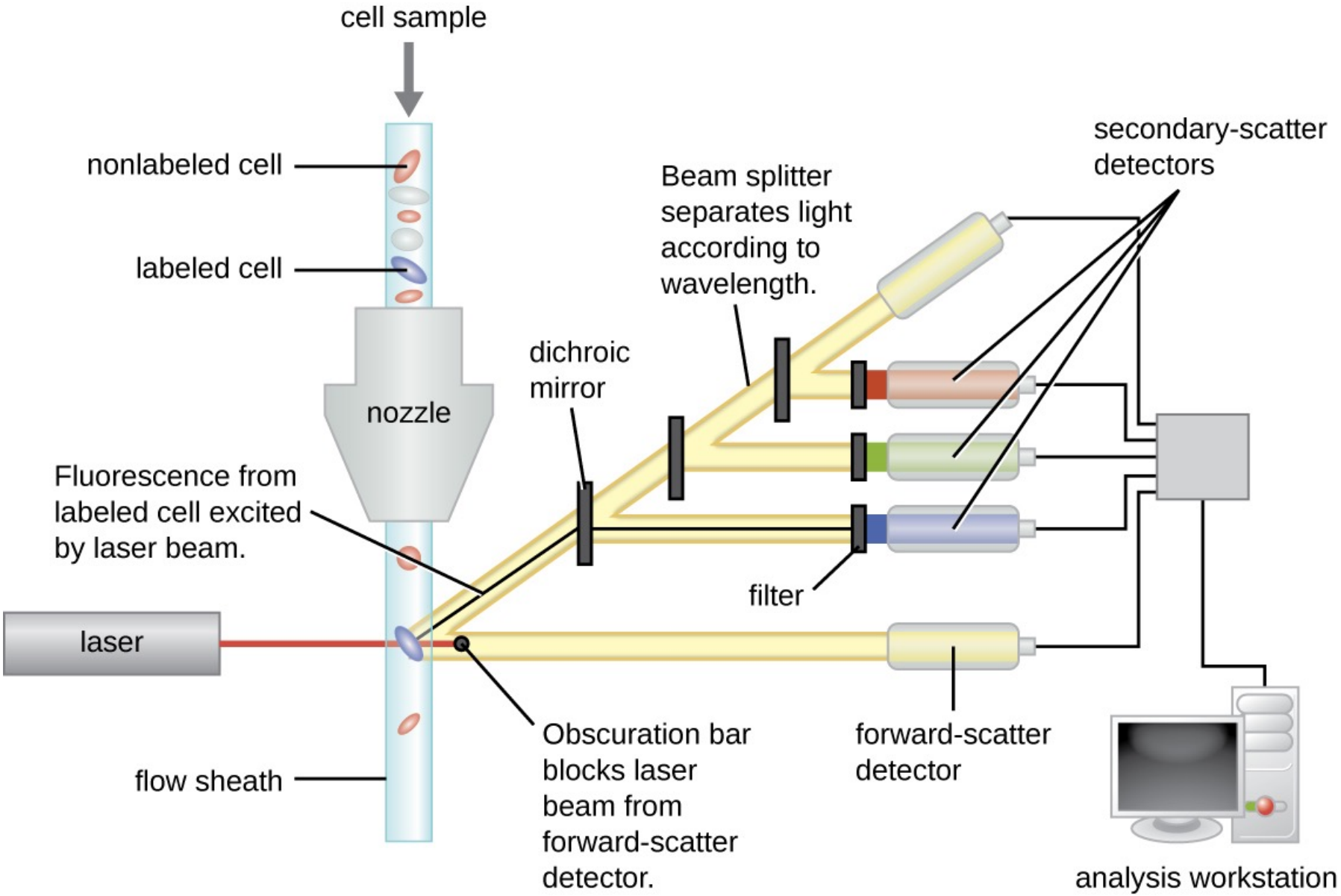


Fig. 17.1 Schematic diagram of basic principle of flow cytometry is highlighted in the picture. The single cells are hit by the laser beam of particular wavelength. The emitted light is detected by the photomultiplier tube and is converted into a digital pulse. The result of the events is expressed as dot plot or histogram


cell sample
nonlabeled cell
labeled cell
nozzle
Fluorescence from labeled cell excited by laser beam.
laser
flow sheath
Obscuration bar blocks laser beam from forward-scatter detector.
dichroic mirror
Beam splitter separates light according to wavelength.
secondary-scatter detectors
filter
forward-scatter detector
analysis workstation

Fluorochrome Dyes Used used in FCM

Fig. 17.2 The diagram shows the principle of fluorochrome dye activation. When a fluorochrome compound absorbs quantum of light, the electron moves from lower orbit to the higher orbit, and the compound remains in excited state. When the compound comes back to its ground state, the electron returns to its original orbit and the compound emits the quantum of light of lower wavelength with different colour

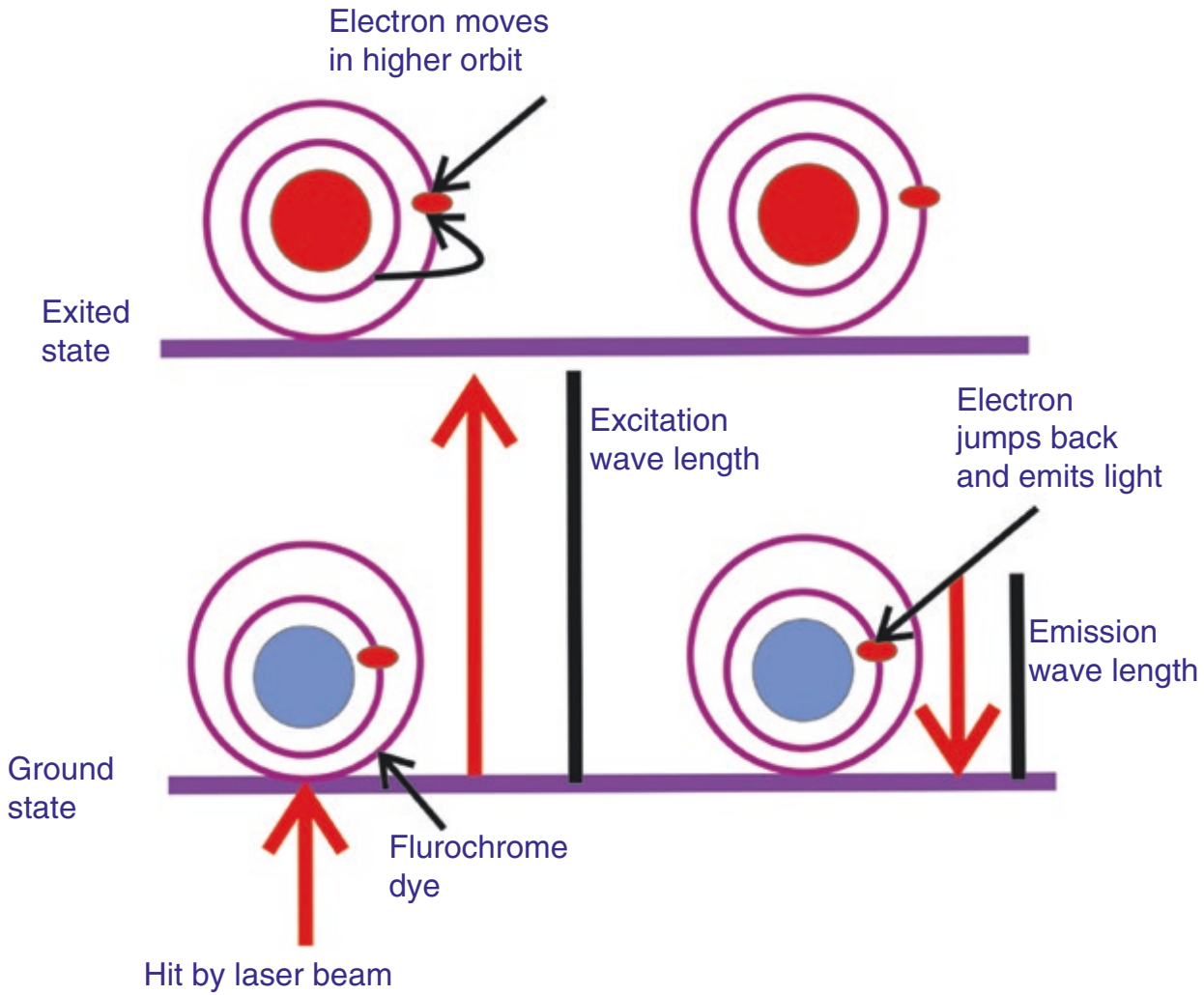


Table 17.1 Fluorochrome dye used in DNA

Flurochrome	Excitation maximum (nm)	Emission maximum (nm)
Propidium iodide	305, 540	620
Ehidium bromide	493	620
Hoechst 33342	350	461
Hoechst 33258	352	461
Diamidinophenylindole (DAPI)	359	461
Acridine orange	503	530 (DNA), 640 (RNA)

Table 17.2 Fluorochrome dye for conjugating with antibody

Fluorochrome	Excitation maximum (nm)	Emission maximum (nm)
Fluorescein isothiocyanate (FITC) 488	495	519
Phycoerythrin (PE)	496	576
Allophycocyanin (APC)	650	660
Rhodamine Red-X	570	590
Texas Red®	595	613
PE-Cy7® 488	566	778
Peridinin chlorophyll (PerCP)	477	678

nm nano micrometre, 10^{-9} m

Samples for Flow Cytometry

Cytology Samples

- The various types of cytology specimens for FCM include:
- 1. Fine needle aspiration cytology materials: Lymph node, breast, lung, prostate, etc.
- 2. Exfoliative samples: Effusion fluid, CSF, bladder wash

Advantages of Cytology Sample for FCM

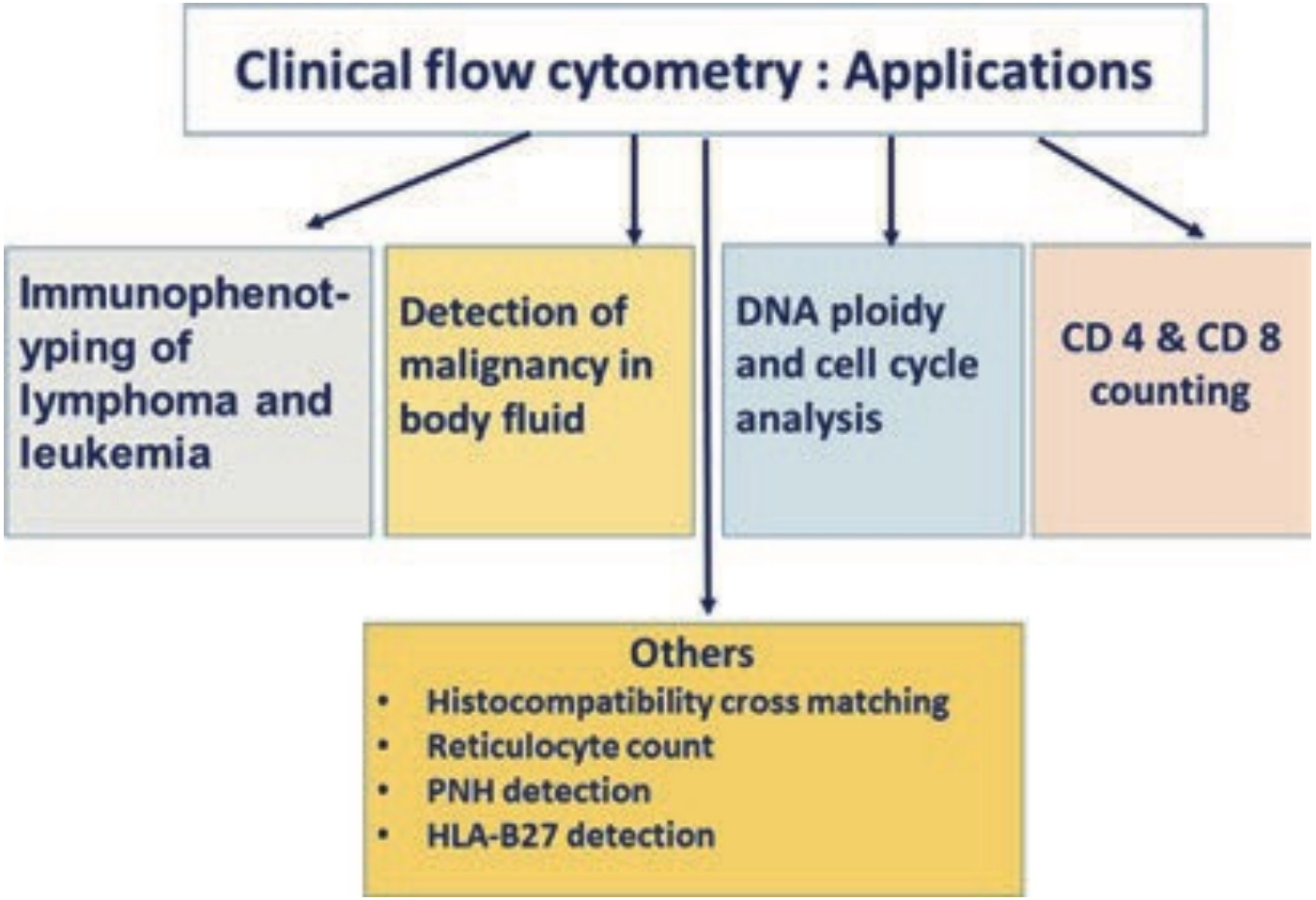
- Easy to process
- Less effort to disaggregate the cells
- Easy to procure the sample
- Possible to study from the multiple areas of the tumor or lymph nodes
- Viable cells so functional studies are possible

Histology Samples

1. Frozen section tissue
2. Paraffin-embedded tissue

- Histopathology sample needs thorough disaggregation for flow cytometry.
- In fact, paraffin-embedded tissue does not give good result on FCM.

Targets of Applications



Common applications of flow cytometry is highlighted in this diagram

Measurement of Cellular Features

- DNA ploidy analysis
- Cell cycle analysis
- Intracellular and cell surface receptors
- Cell size
- Cell viability and apoptotic cells
- Enzyme activity: phosphatase, glucuronidase, etc.

Measurement of Cellular Features

- Metabolic studies: oxidative burst, intra-cellular pH, intracellular calcium
- Cellular protein, lipid, haemoglobin
- RNA content
- Mitochondrial function
- Physical phenomena: phagocytosis, pinocytosis, etc.

DNA Content & Ploidy Analysis

- DNA-specific dye stoichiometrically binds with the DNA of the nucleus.
- Emitted fluorescence from the dye-DNA complex is directly proportional to the DNA content of the nucleus.
- The data are displayed as DNA histogram.
- The majority of the normal cells contain diploid (2n) chromosome so they emit a certain amount of fluorescence represented by a channel number. This forms a single peak known as the diploid peak in DNA histogram.
- The cells in G2M phase contain the double amount of DNA (4n) so they emit the double amount of fluorescence and are present in double channel number in the DNA histogram forming a small tetraploid peak. In between these two peaks, the cells have varying amount of DNA from 2n to 4n, and they are in synthetic (S) phase.
- Any peak, other than these two peaks is considered as an aneuploid peak.

- The following information is obtained from DNA FCM:
- 1. Identification of aneuploid cell population
- 2. DNA index
- 3. Proliferative cell fraction (% of S phase)
- **DNA Ploidy** The clone of cells containing the abnormal amount of DNA is known as aneuploid cells.
- In DNA histogram the aneuploid population will form a peak somewhere other than diploid peak.

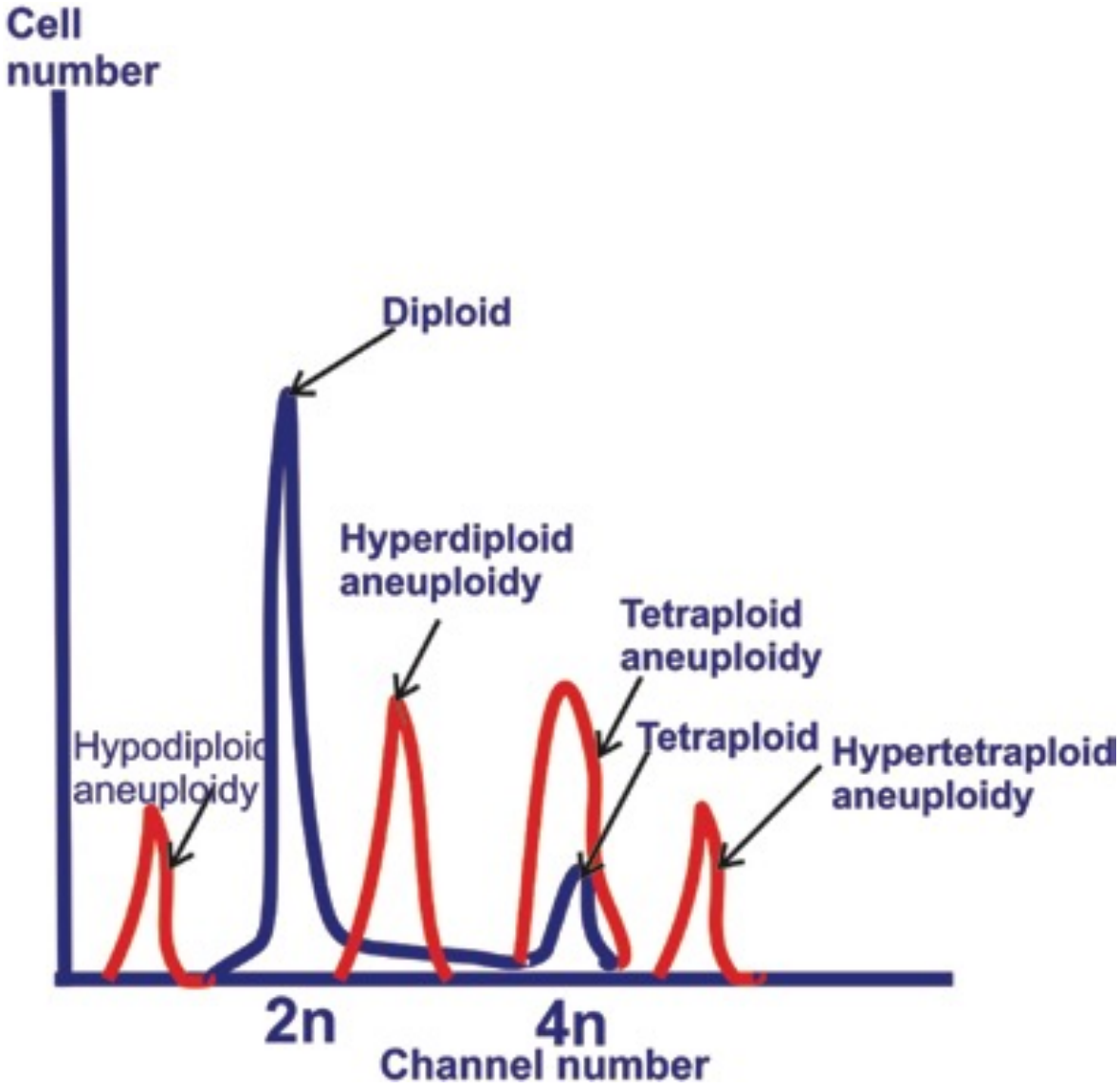
The relative DNA content of the aneuploid population of cells is calculated by DNA index.

$$\text{DNA index} = \frac{\text{Mean channel number of aneuploid peak}}{\text{Mean channel number of normal diploid peak}}$$

The different types of aneuploidy are mentioned below:

- **Hyperdiploid Aneuploidy.** Here the aneuploid peak lies in between diploid and tetraploid peak (DNA index is in between 1 and 2).
- **Hypodiploid Aneuploidy.** Here the aneuploid cell population forms a peak left to the diploid peak as they contain less than the 2n amount of DNA (DNA index is lower than 1).

- **Tetraploid Aneuploid.** Aneuploid population of cells makes a peak on G2M peak. It is often difficult to distinguish tetraploid peak from normal G2M peak. However, the presence of more than 20% cell population in this peak suggests a tetraploid aneuploidy (DNA index is 2).
- **Hypertetraploid Aneuploidy.** Here the aneuploid population of cells forms a peak beyond the G2M peak as they have more than 4n amount of DNA peak (DNA index is greater than 2).
- **S Phase** The S-phase fraction of cells has 2n–4n amount of DNA so they remain in between G0G1 and G2M peak. The number of such cells can be calculated from the DNA histogram.



Schematic diagram of different types of aneuploidy in DNA histogram

Clinical Application

- DNA FCM provides usual information on the DNA ploidy and S-phase fraction of the tumour cell population.
- The presence of aneuploidy cell population and/or high S phase suggests a malignant lesion.
- However, we should keep in mind that malignant tumours may often show diploid cell population, and also uncommonly benign tumour may have aneuploidy population of cells.

Clinical Application.,

- **Diagnosis**
- DNA FCM along with staining of other markers (BER EP4, Leu M1, etc.) helps to identify adenocarcinomas in effusion fluid.
- DNA ploidy estimation in the gated population of cytokeratin positive cell is helpful in the detection of malignancy in follow-up cases of urothelial carcinoma of the bladder.

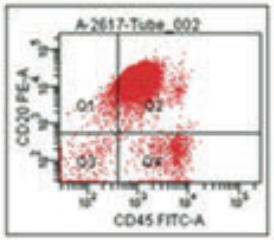
Clinical Application.,

- **Prognosis of the Patients**
- DNA aneuploidy and high S phase are poor prognostic factors in various solid tumours such as bladder, prostate, ovarian and endometrial carcinomas.
- **Immunophenotyping**
- A large number of CDs have been described in different cells of lymphoid origin.
- The demonstration of various CD markers on the lymphoid cell surface helps in the diagnosis and also sub-classification of lymphomas.

Table 17.3 CD markers of lymphoid cells

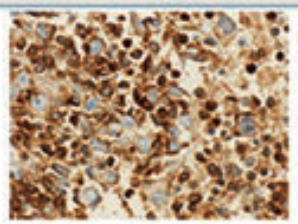
Cells	Markers
All lymphoid cells	CD 45
T lymphocyte	CD 2 CD 3
T helper cell	CD 4
T cytotoxic cell	CD 8
T regulatory cell	CD 4 CD 25
NK cell	CD 56
B lymphocytes	CD 19 CD 20
Plasma cell	CD 138
Plasmacytoid dendritic cell	CD 304
Stem cell and progenitor cell	CD 34 CD 117 CD 271

Flow cytometric immunophenotyping



- Rapid
- More objective
- Large number of CD markers can be used
- Quantitation of CD positive cell possible
- Quantitation of intensity of antigen positivity possible
- Morphology of the cells not possible to see
- No archival storage

Immunohistochemistry



- Slow process
- Less objective
- Limited number of CD markers can be used
- Manual quantitation of CD positive cell possible
- Quantitation of intensity of antigen positivity not possible
- Morphology of the cells can be seen
- Archival storage possible

flow cytometric immunostaining versus immunocytochemistry

Limitations of FCI

- ***No morphological correlation***: It is not possible to correlate morphological findings with the FCI data. Laser scanning cytometry overcomes this problem.
- ***Admixture of other material***: Benign reactive components of the lymph node such as vascular or stromal material are often admixed with the cells of interest, and appropriate gating is needed to eliminate such cells.
- ***Scanty cells may be missed***: It is difficult to identify Reed-Sternberg cell in the specimen of Hodgkin's lymphoma because of scanty R-S cells in the specimen.
- ***No light chain restriction***: All cases of B-NHL may not always show light chain restriction. This may be due to failed expression of light chain on the surface of the cell.
- ***High cost of FCM instrument***: FCM is overall a costly technique and needs good skill.

References & Credits

- Pranab Dey, (2018), ***Basic and Advanced Laboratory Techniques in Histopathology and Cytology***, Springer Nature Singapore Pte Ltd.

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End of Lecture

Cellular Pathology