Flow Cytometry

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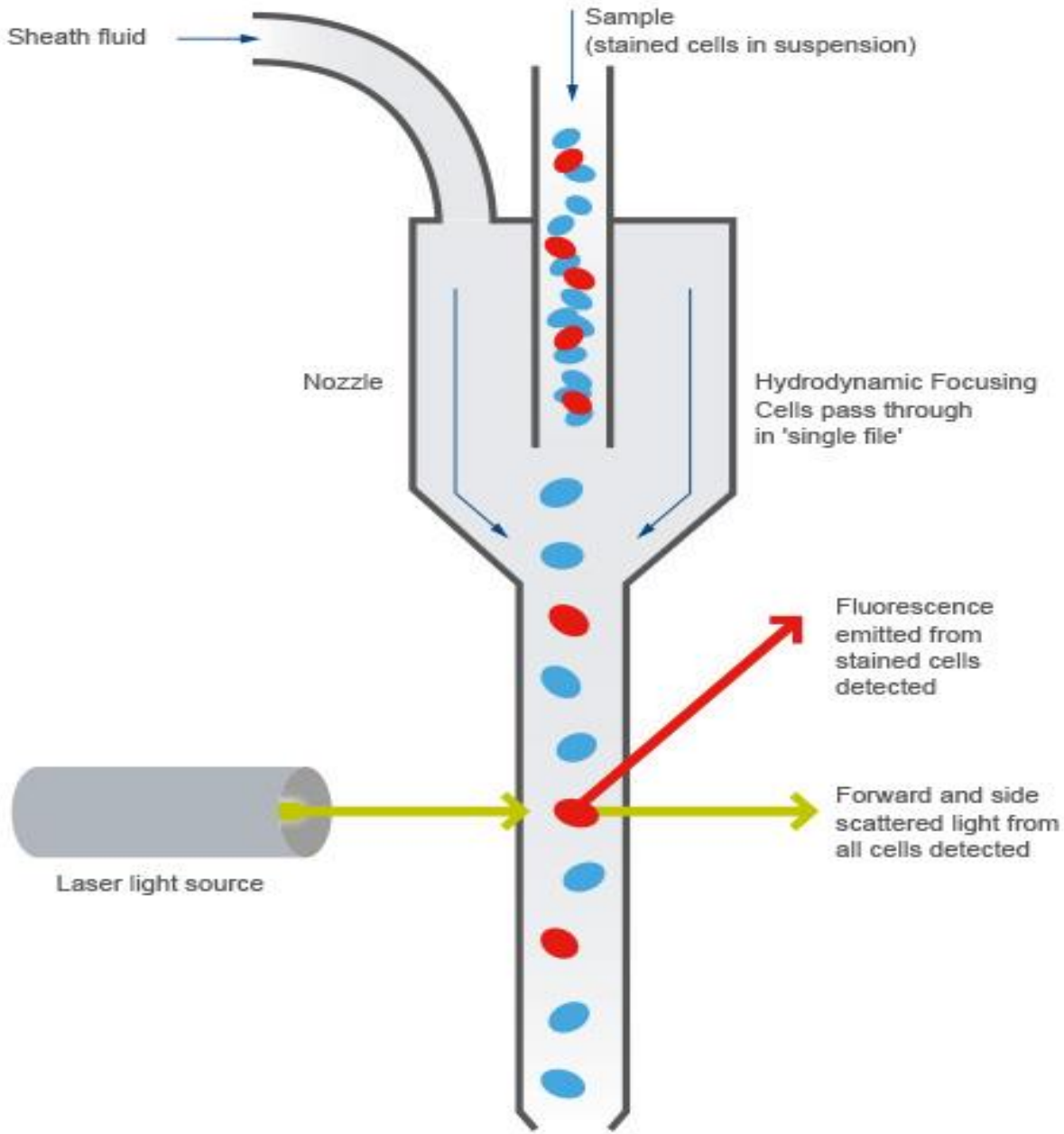
- **What is flow cytometry?**

Flow cytometry is a popular laser-based technology to analyze the characteristics of cells or particles.

It is predominantly used to measure fluorescence intensity produced by fluorescent-labeled antibodies detecting proteins, or ligands that bind to specific cell-associated molecules.

Flow cytometry is a method of measuring multiple physical and chemical characteristics of particles by optical means.

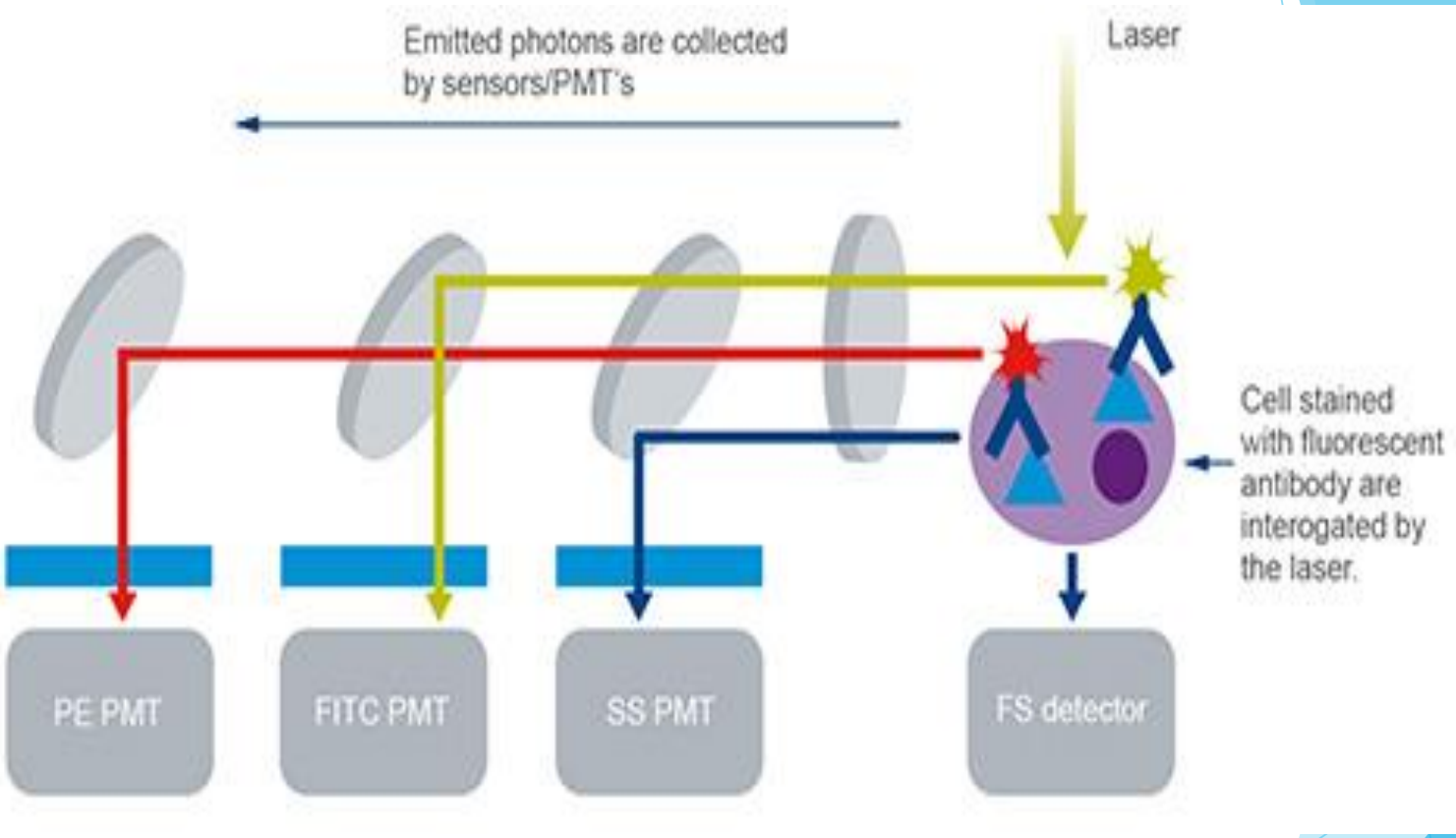
Flow Cytometry



What can a Flow Cytometer tell us about a particle?

- Its relative size

  (Forward Scatter: FSC)
- Its relative granularity or internal complexity (Side Scatter: SSC)
- Its relative fluorescence intensity


Emitted photons are collected by sensors/PMT's
Laser
Cell stained with fluorescent antibody are interogated by the laser.
PE PMT
FITC PMT
SS PMT
FS detector

A Flow Cytometer needs a combined system of:

Fluidics

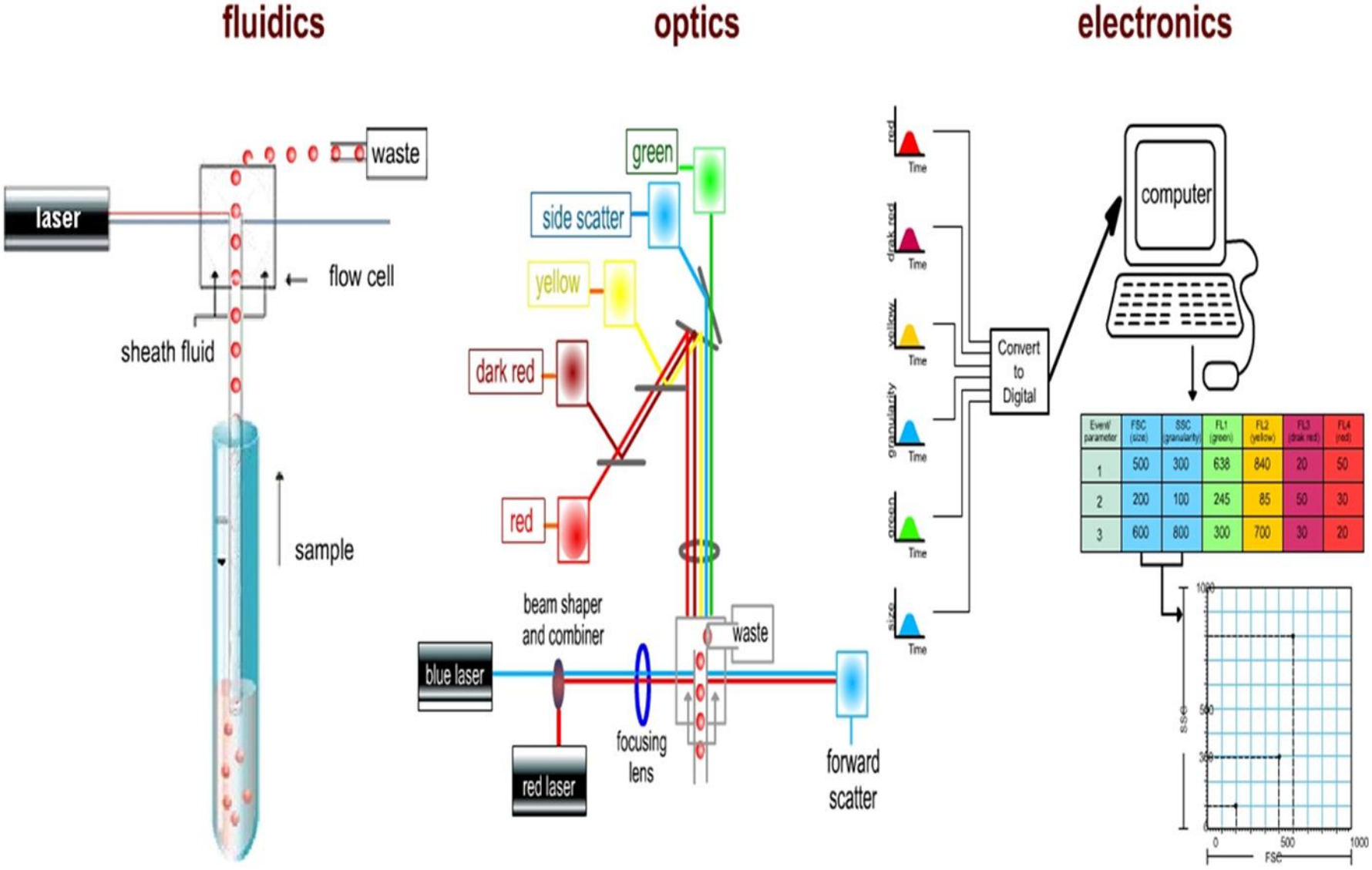
- To introduce and focus the cells for interrogation

Optics

- To generate and collect the light signals

Electronics

- To convert the optical signals to proportional electronic signals and digitize them for computer analysis

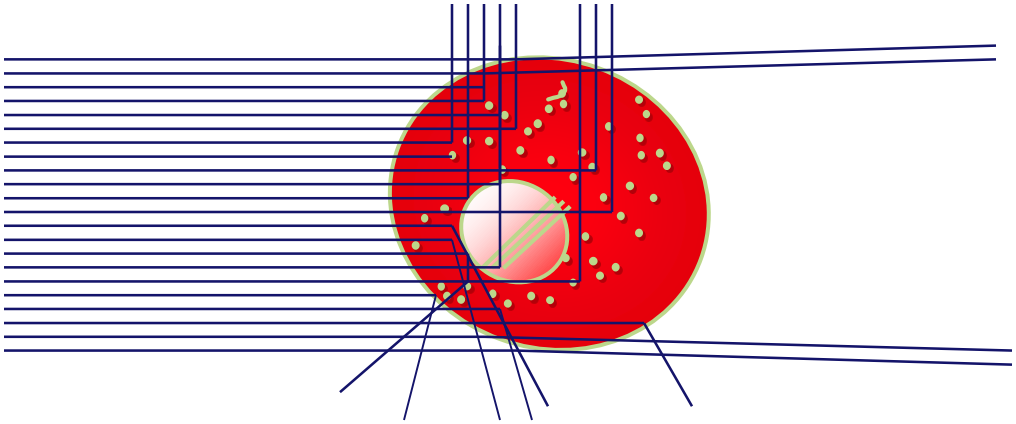

fluidics
optics
electronics
waste
laser
flow cell
sheath fluid
sample
green
side scatter
yellow
dark red
red
beam shaper
and combiner
blue laser
red laser
focusing
lens
waste
forward
scatter
Time
Convert
to
Digital
computer
Event parameter
FSC (size)
SSC (granularity)
FL1 (green)
FL2 (yellow)
FL3 (drak red)
FL4 (red)
1
500
300
638
840
20
50
2
200
100
245
85
50
30
3
600
800
300
700
30
20
SSC
FSC
0
500
1000

Properties of FSC and SSC

Right Angle Light Detector, Cell Complexity

3° Forward Light Detector, Cell Surface Area

Incident Light Source

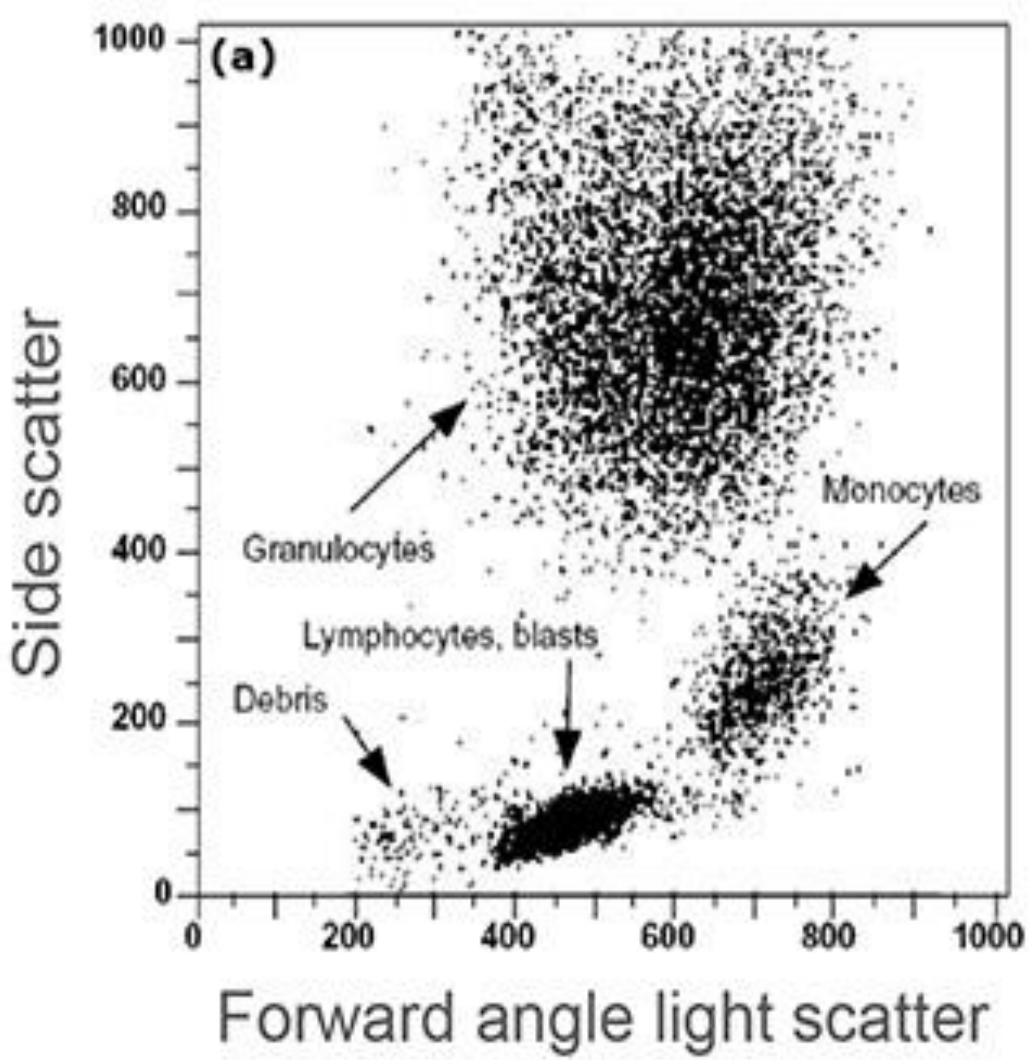


Forward Scatter—diffracted light

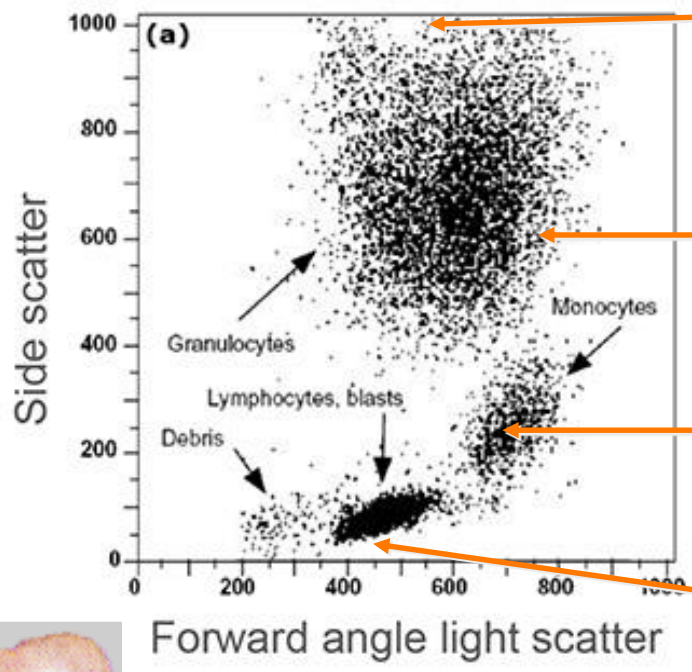
- Related to cell surface area
- Detected along axis of incident light in the forward direction

Side Scatter—reflected and refracted light

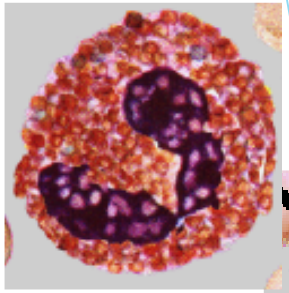
- Related to cell granularity and complexity
- Detected at 90° to the laser beam


(a)
Side scatter
Forward angle light scatter
1000
800
600
400
200
0
0
200
400
600
800
1000
Granulocytes
Monocytes
Lymphocytes, blasts
Debris

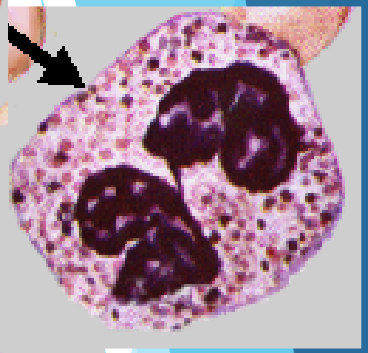
Morphology vs. FSC to SSC dot plot



Eosinophils

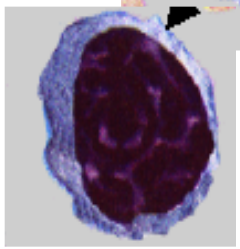


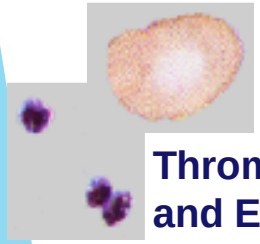
Neutrophils



Monocytes

Lymphocytes





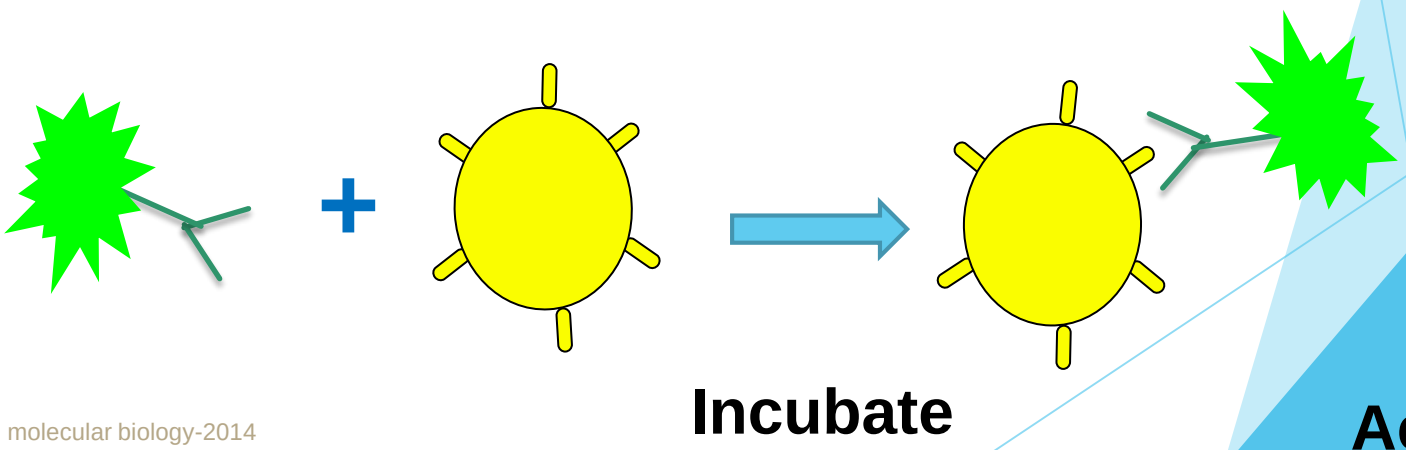
Thrombo and Ery. and Debris

Phenotyping, Size and Granularity detection:

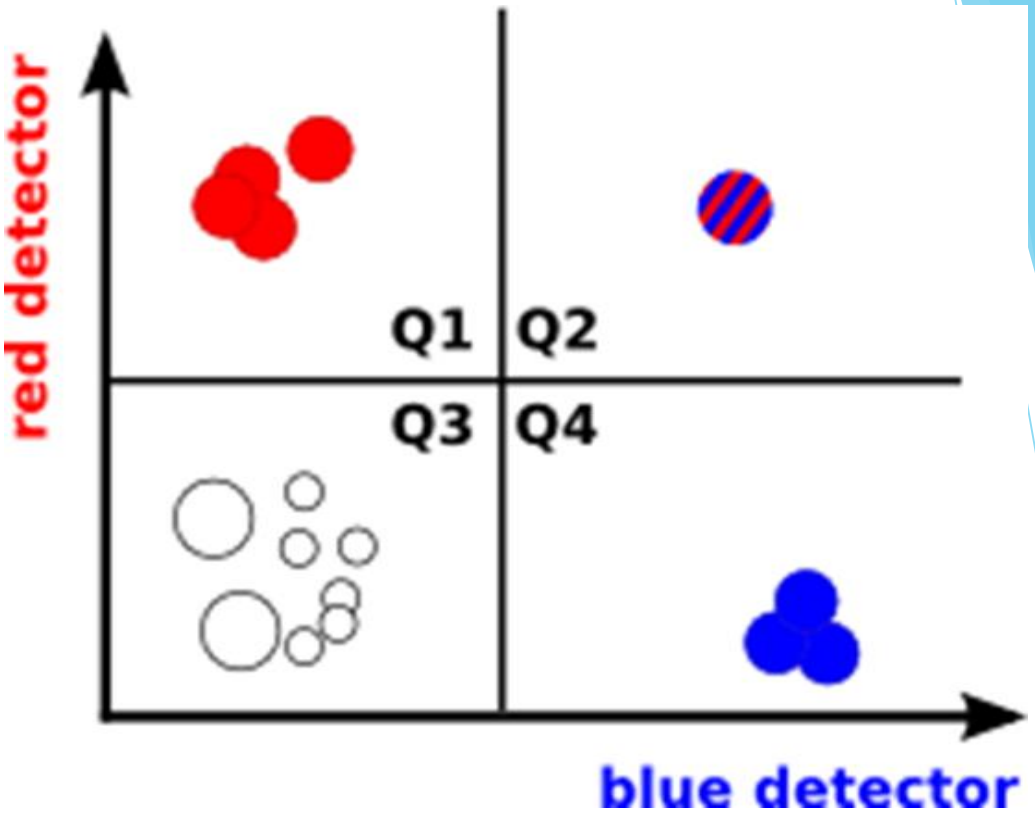
Sample requirements:

A suspension of single cells or other particles in a suitable buffer, usually PBS.

Typical density : 10^5 - 10^7 cells / ml



Dot plot



Q1: Single positive for the red detector +

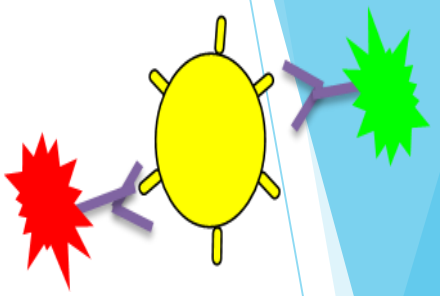
Q2: Double positive for red and blue ++

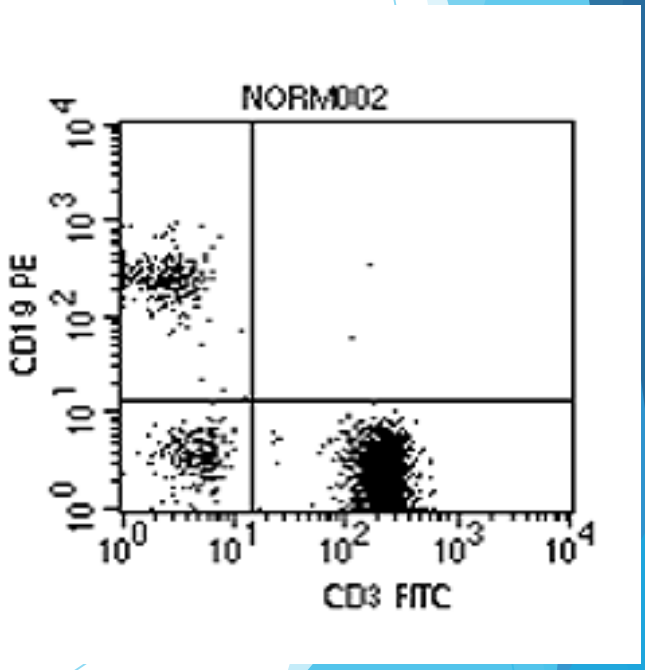
Q3:Double Negative for red and blue ++

Q4: Single positive for the blue detector +

Dot-plot

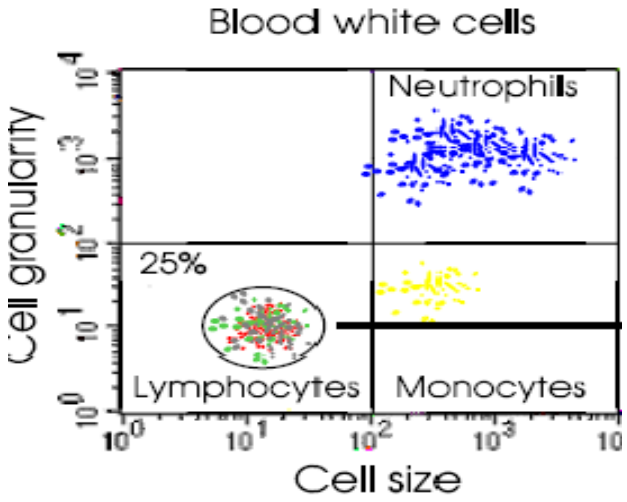
- The most useful when you need to compare multiparametric data (e.g. Intensity of side-scatter vs forward-scatter channels).
- Can be two- or three-dimensional
- Intensity of each channel is represented on its own axis.
- Each distinct event is represented at a single dot.



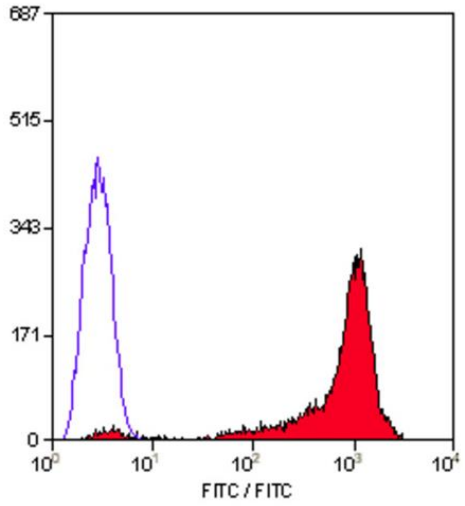


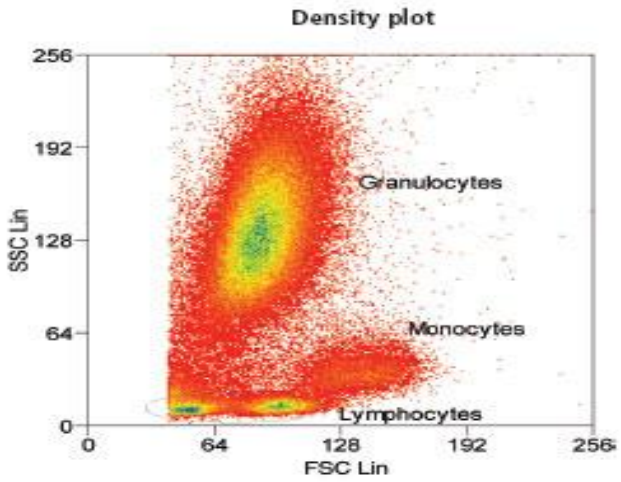
Data Analysis: plot types

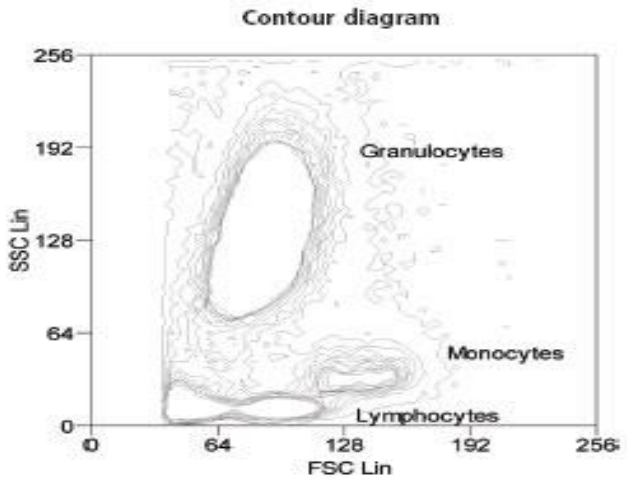
Dot plot



Histogram

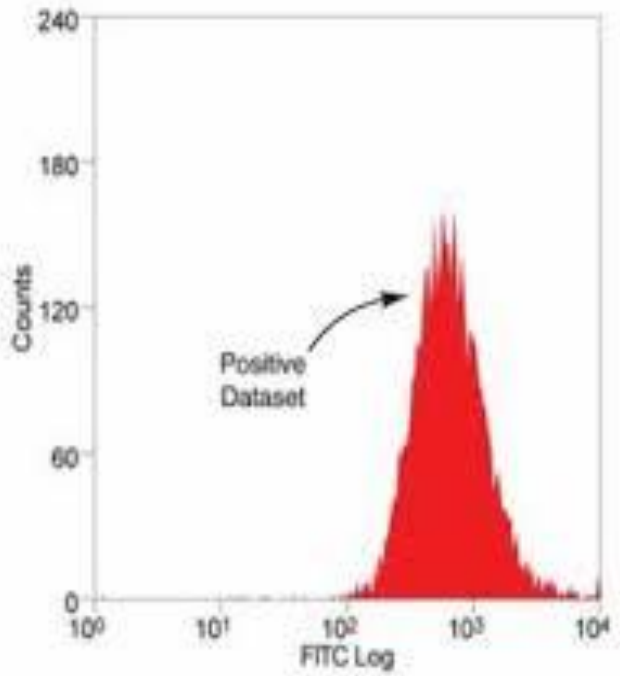


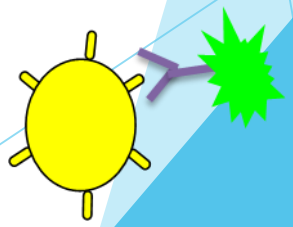


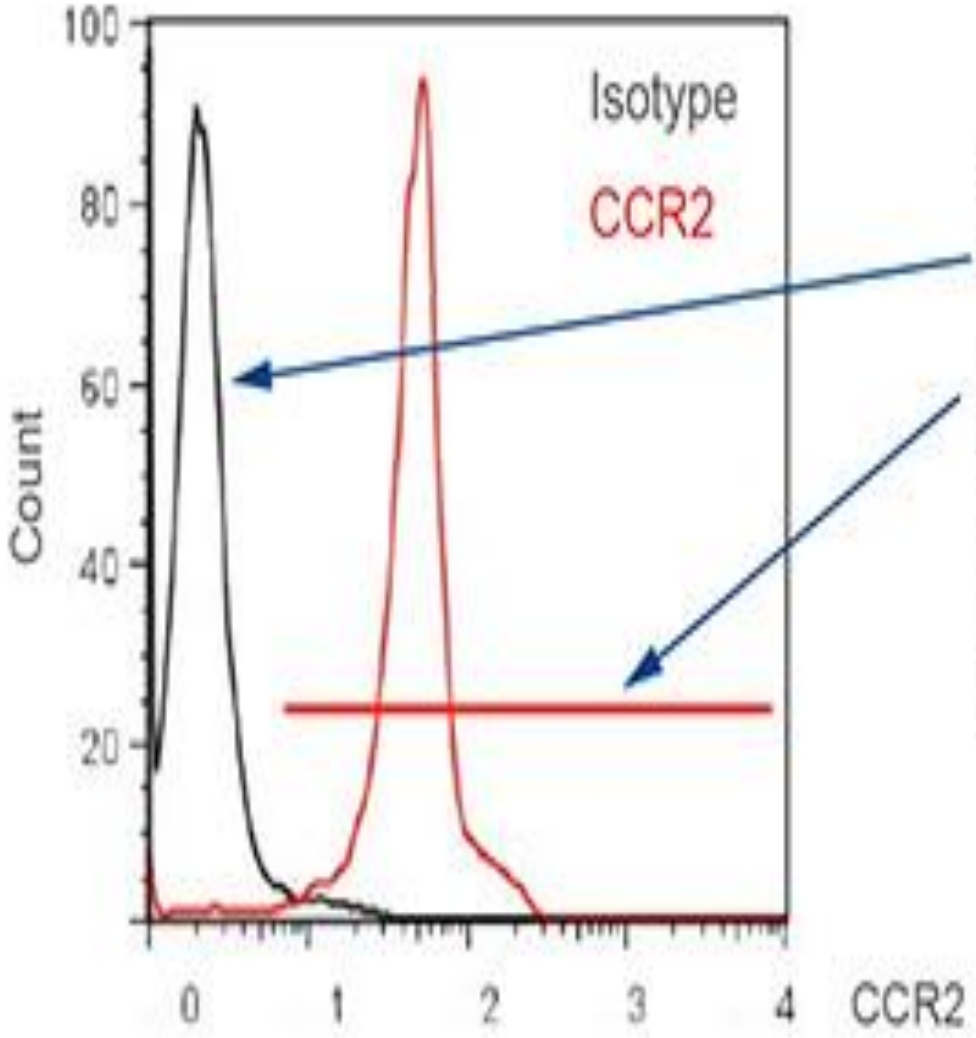


Histogram

- Fast to read and easy to understand.
- The most useful when only one parameter (e.g. intensity from a single fluorescent channel) is important.
- Usual representation includes the intensity of a single channel (horizontal axis) vs number of detected events (vertical axis).







Negative control / isotype control peak in first log decade

Positive cell population.
Stained cells give shift (increase) in fluorescence intensity to the right.
Anything within the gate marked here is a positive event.

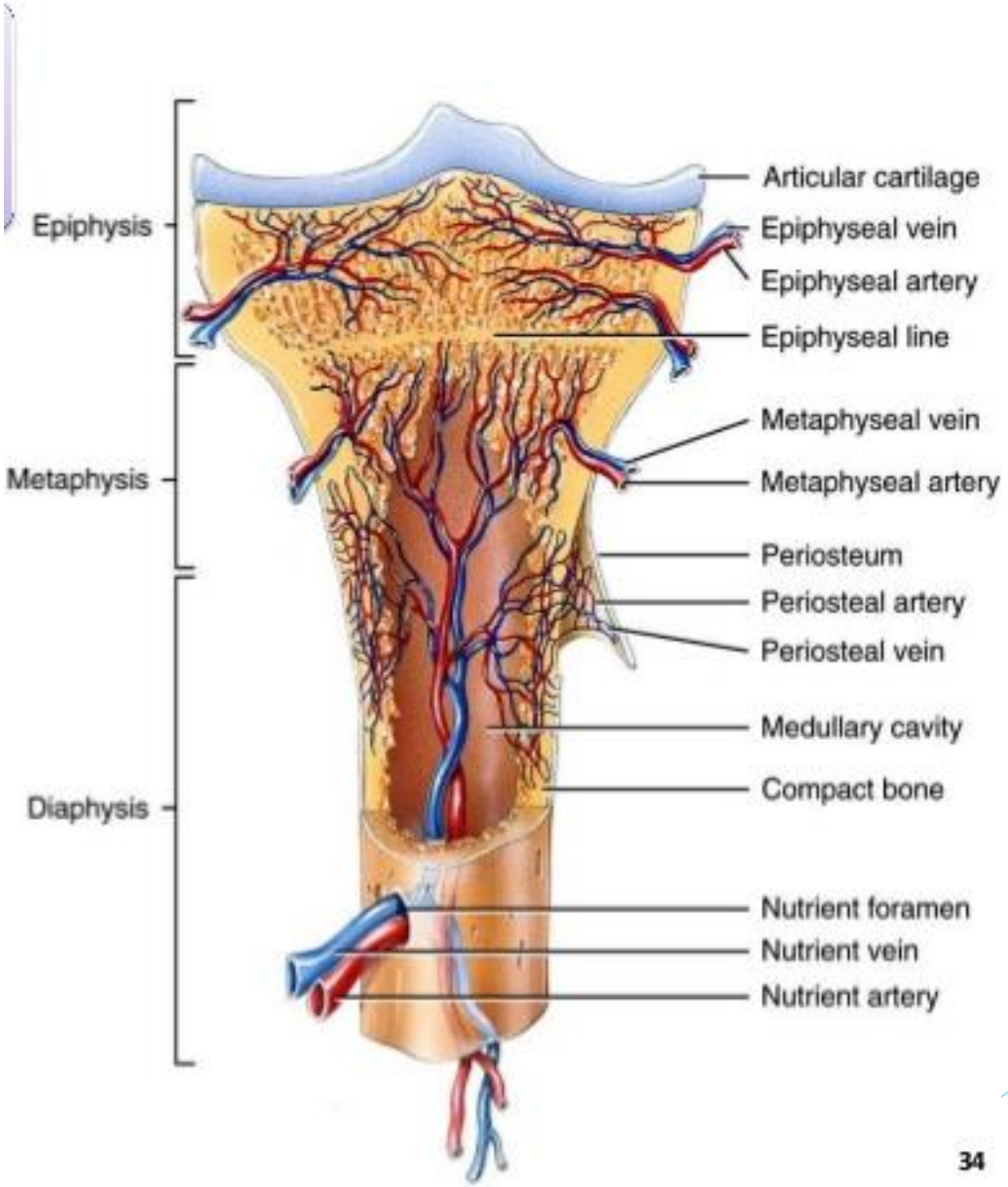
Bone Marrow Histology

- The term ‘bone marrow’ (BM) refers to the tissue occupying the cavities under the cortex within the honeycomb of trabecular bone.
- The BM is composed of:
  - The parenchyme: the hemopoietic cells including precursors and mature cells of the erythroid, myeloid and megakaryocytic lineages, i.e. various stages of red cells, white cells, megakaryocytes, lymphocytes, plasma cells and mast cells
  - The stroma: fat cells, histiocytes/macrophages, fibroblasts, blood vessels and intercellular matrix.

Bone marrow structure

- The constituents of the normal BM are closely packed within a hard bony ‘container’.
- Hemopoiesis occurs in the intertrabecular space within marrow cavities.
- The bony trabeculae (cancellous bone) are lined by endosteum, osteoblasts and osteoclasts.
- The stromal elements form an extensive, closely woven network in which the hematopoietic precursors are embedded, attached in various ways and to different components by the adhesive proteins and by other cells, such as the central macrophages in the erythroid islands.

- The blood supply to the BM consists of two systems: periosteal arteries, which give off branches to the BM after they penetrate the bone, and nutrient arteries.
- Blood drains from the BM cavity through central veins.


Epiphysis
Metaphysis
Diaphysis
Articular cartilage
Epiphyseal vein
Epiphyseal artery
Epiphyseal line
Metaphyseal vein
Metaphyseal artery
Periosteum
Periosteal artery
Periosteal vein
Medullary cavity
Compact bone
Nutrient foramen
Nutrient vein
Nutrient artery

- The microvasculature of the BM comprises a network of sinusoids.
- Hemopoiesis only occurs in the interstital space between these sinusoids, thereby ensuring that hemopoietic progenitors are located close to the blood supply.

Bone marrow trephine biopsy

Staining

- Giemsa staining generally gives better discrimination of cell types based on cytoplasmic staining characteristics, that is:

1. myeloid/eosinophil granules: red
2. mast cell granules: purplish red
3. erythroid precursor cytoplasm: blue
4. plasma cell cytoplasm: purplish blue
5. perinuclear Golgi complex also seen as a pale area.

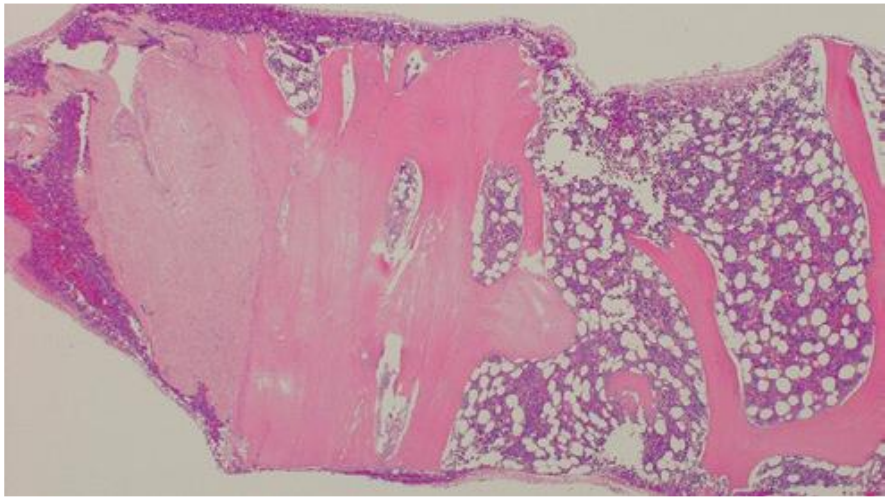
- Other stains commonly used include:
  - the Perls stain for iron
  - silver stains for reticulin
  - Masson’s trichrome stain for collagen.

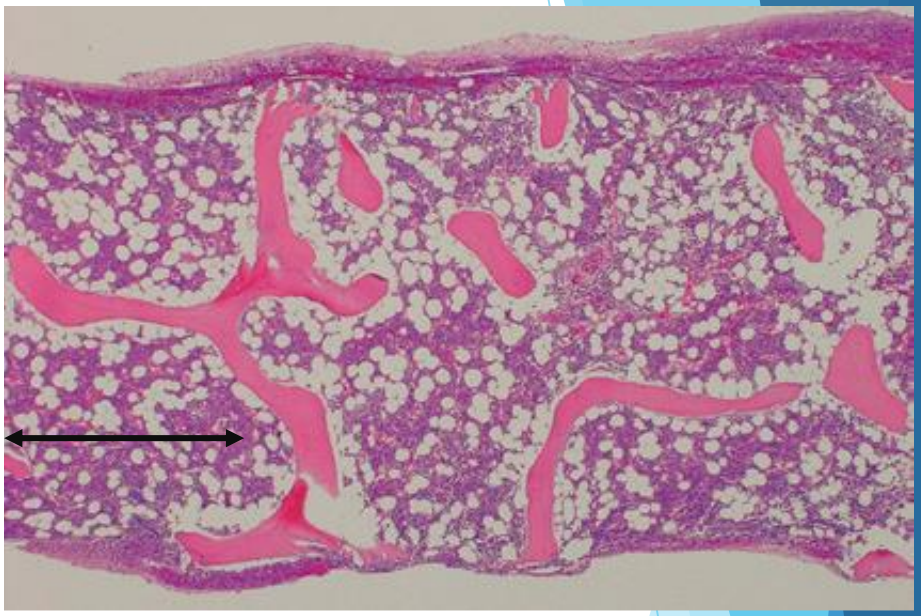
Cellularity ranges for various age groups

Age	Cellularity
Newborn to 3 months	80–100%
Childhood	60–80%
20–40 years	60–70%
40–70 years	40–50%
>70 years	30–40%

Marrow architecture

- The outermost elements of the biopsy are composed of collagenous periosteal connective tissue, followed by a zone of cartilage or cortical bone (depending on the age of the patient).
- After this the bone breaks up into a meshwork of trabeculae, between which are the intertrabecular spaces.
- Hemopoietic cells are present within these intertrabecular spaces and are supported by fat cells, stromal cells, histocytes, extracellular matrix and blood vessels





Low power view of a bone marrow biopsy. (A) Periosteal connective tissue is seen external to cortical bone. (B) Bone trabeculae with the intertrabecular spaces (one marked – arrow) containing hemopoietic cells and fat cells. × 40.

- The hemopoietic cells are located within the intertrabecular spaces.
- The intertrabecular areas can be divided into three zones which contain different hemopoietic cell types:

1. *Endosteal or paratrabecular zone*: immediately adjacent to the trabecular bone and composed predominantly of myeloid precursor cells
2. *Intermediate zone*: contains erythroid colonies and maturing myeloid cells
3. *Central zone*: in the center of the intertrabecular space. In addition to erythroid cells and maturing myeloid cells, this contains sinusoids and megakaryocytes

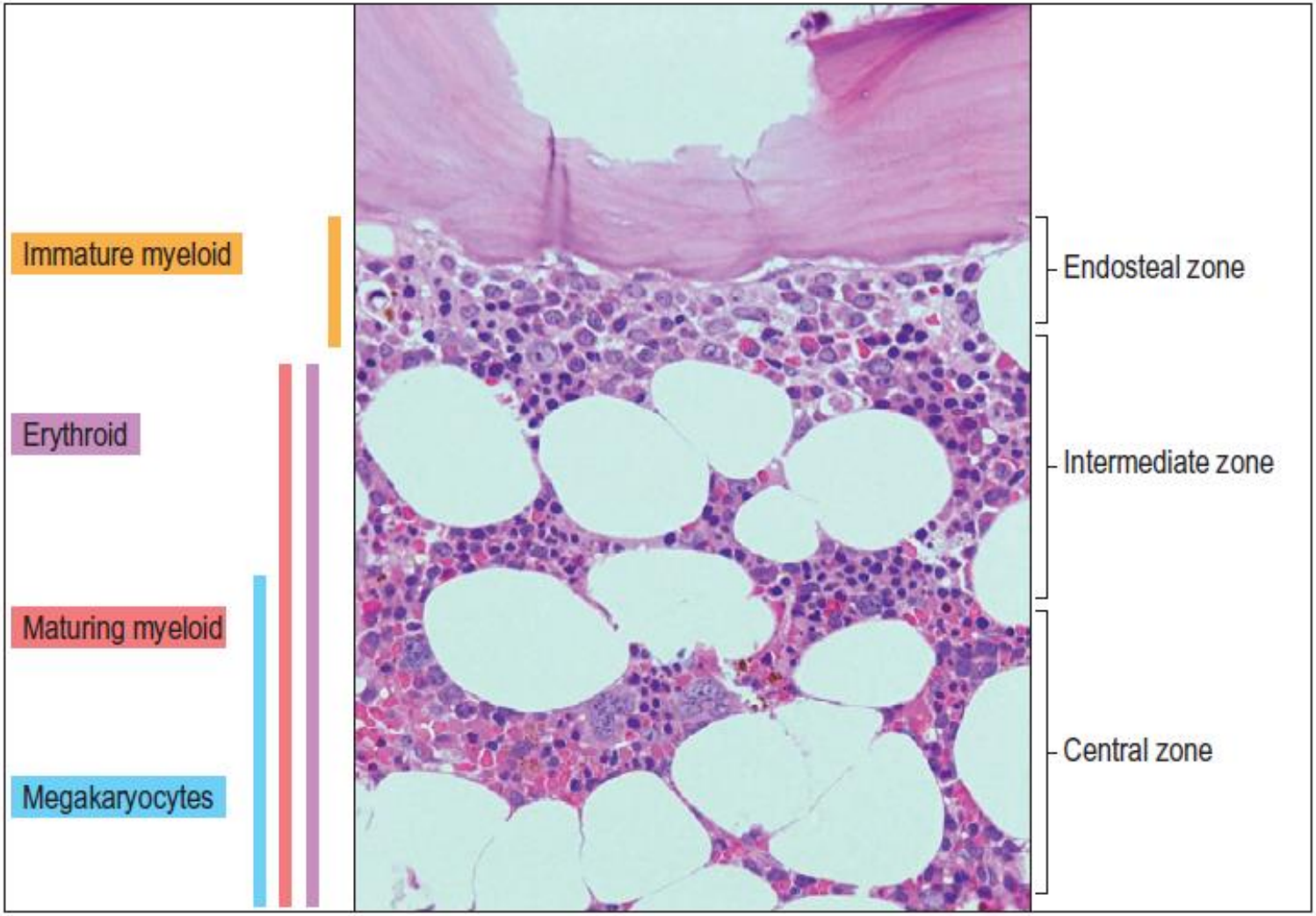


Fig. 3.3 Organization of the bone marrow: zones and distribution of various cell types. ×100.

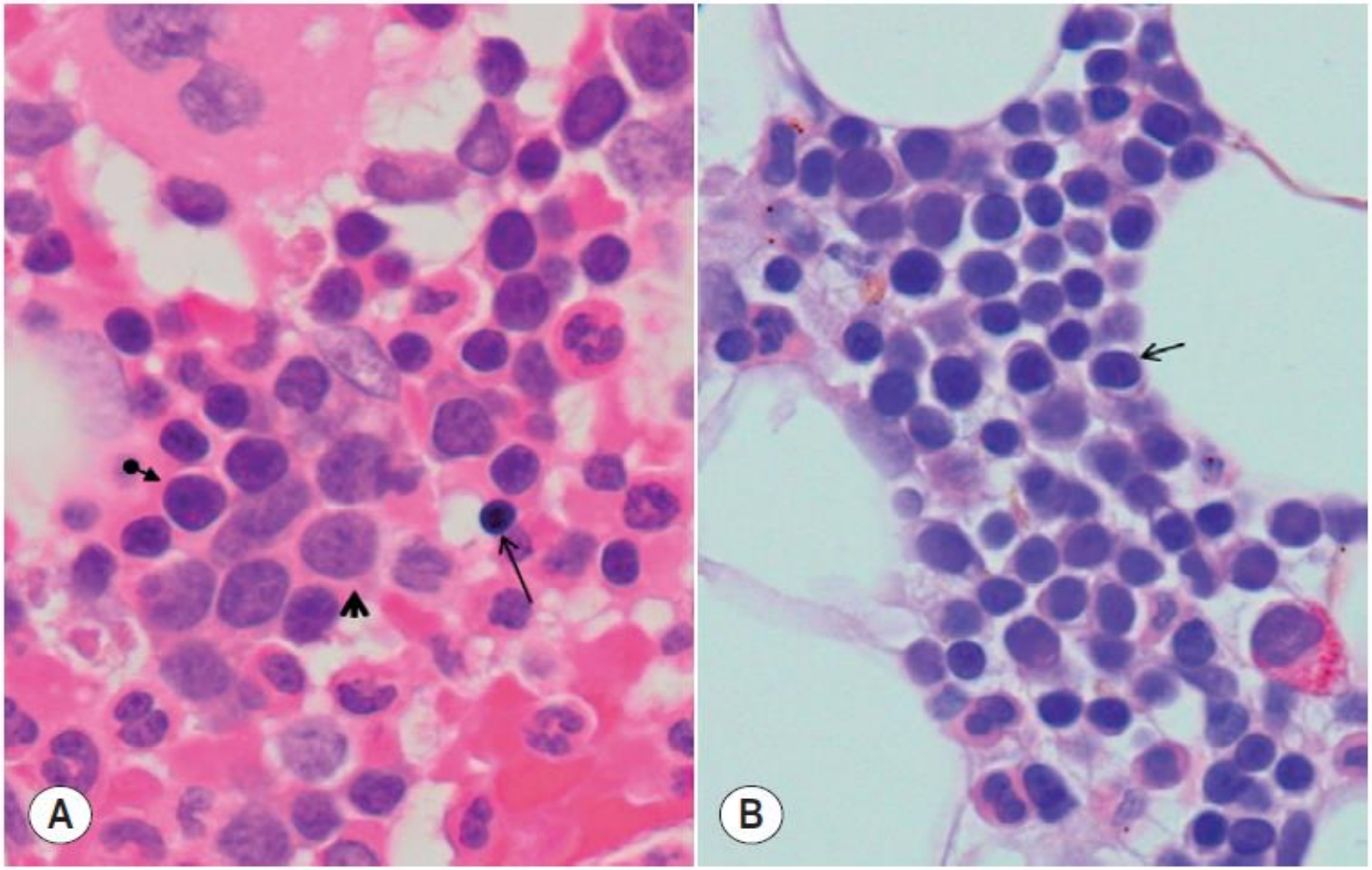


Fig. 3.5 Erythroid cells. (A) Erythroblasts (short arrow), early normoblasts (arrow with a dot) and an extruded erythroid nucleus (long arrow) are seen along with macrophage cytoplasm containing debris. ×400. (B) Erythroid cells showing the round, smooth, hyperchromatic nuclei along with the perinuclear halo artifact (arrow), ×400.

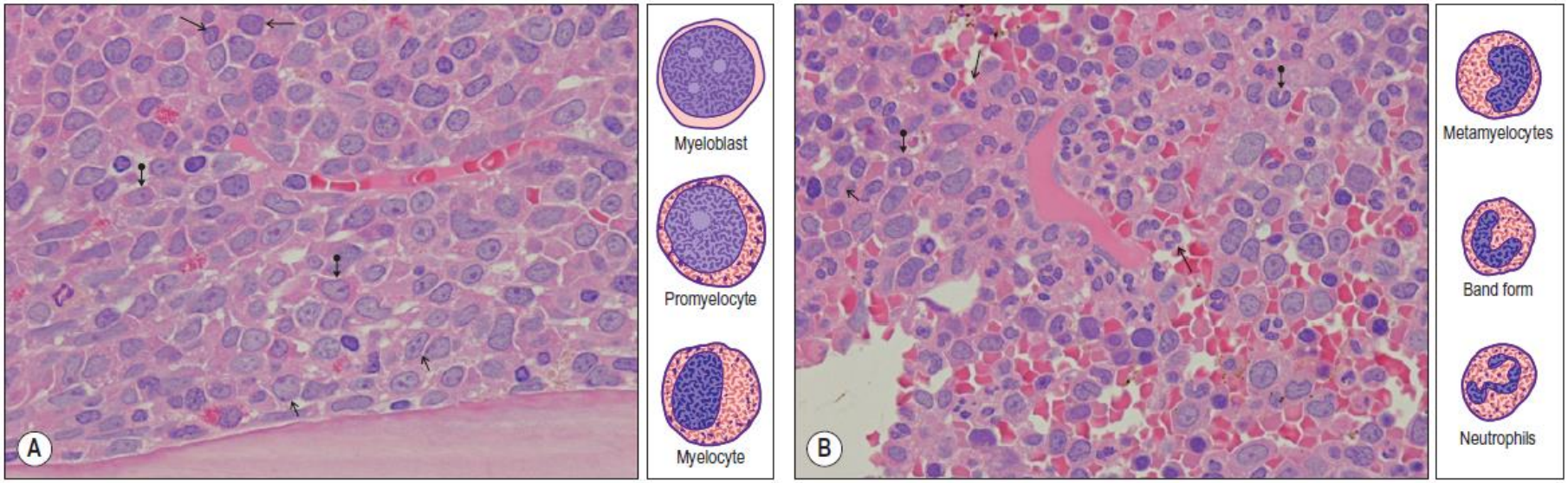
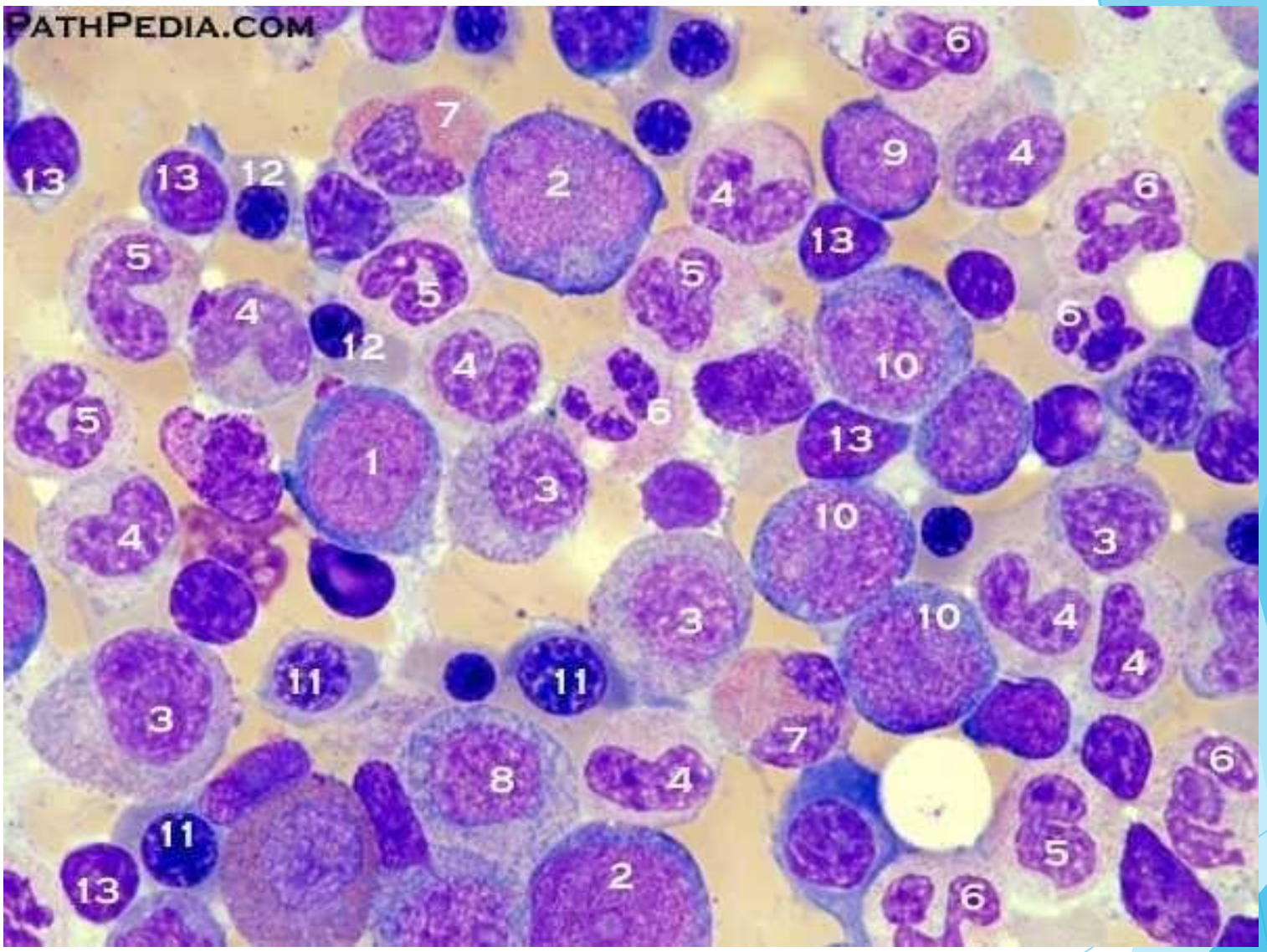
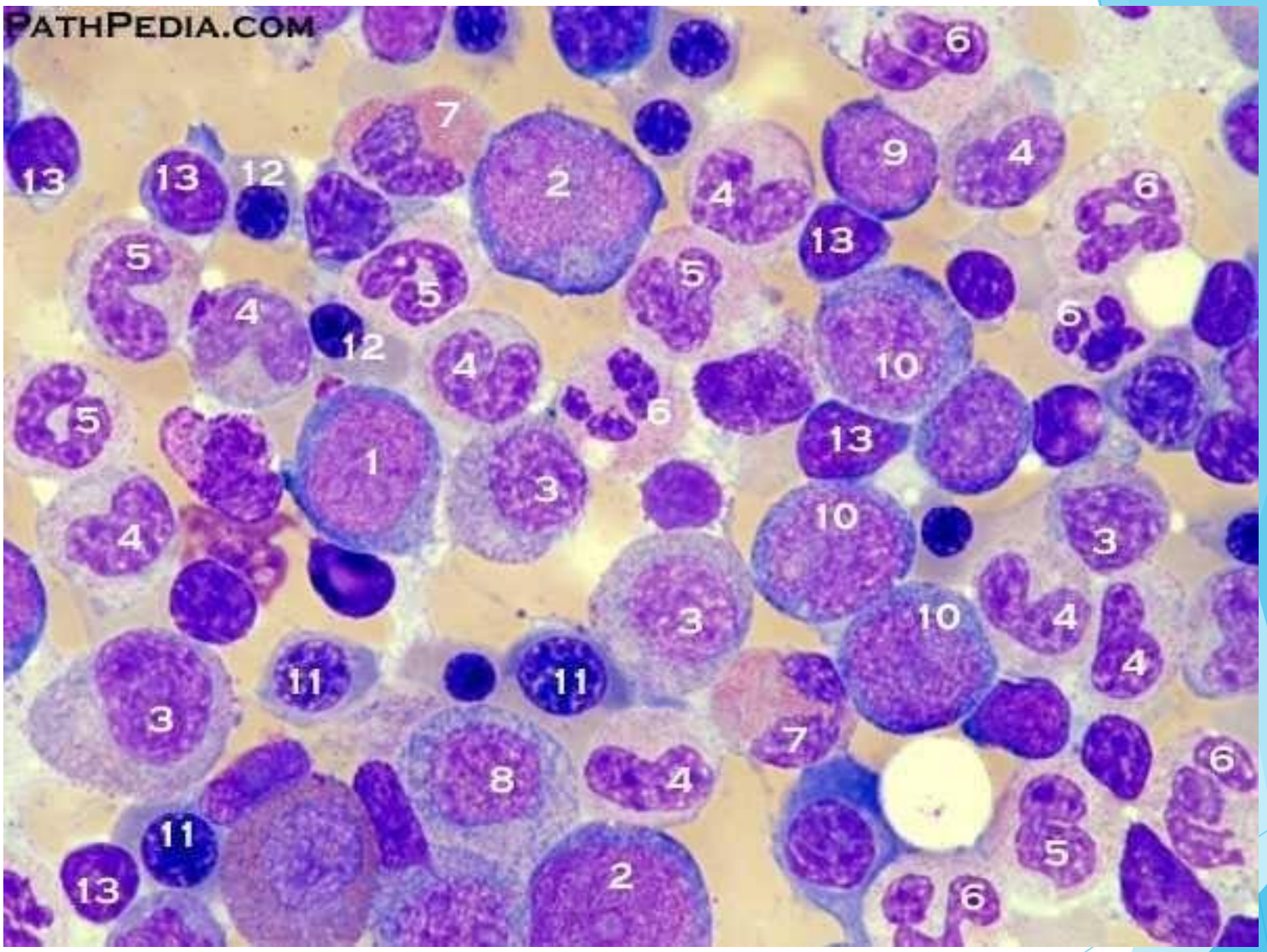


Fig. 3.8 (A) Immature myeloid precursors: myeloblasts (short arrows); promyelocytes (arrows with dots), myelocytes (long arrows). ×600. (B) Late myelopoiesis: metamyelocytes (short arrow); band forms (arrow with dot); neutrophils (long arrows). ×600.



1 = Myeloblast, 2 = Promyeloocyte, 3 = Myelocyte,
4 = Metamyelocytes, 5 = Band neutrophil,
6 = segmented neutrophil



7 = Eosinophil, 8 = Monocyte, 9 =Proerythroblast, 10 = Basophilic erythroblasts, 11 = Polychromatic erythroblast, 12 = Orthochromatic erythroblast, 13 = Lymphocyte.

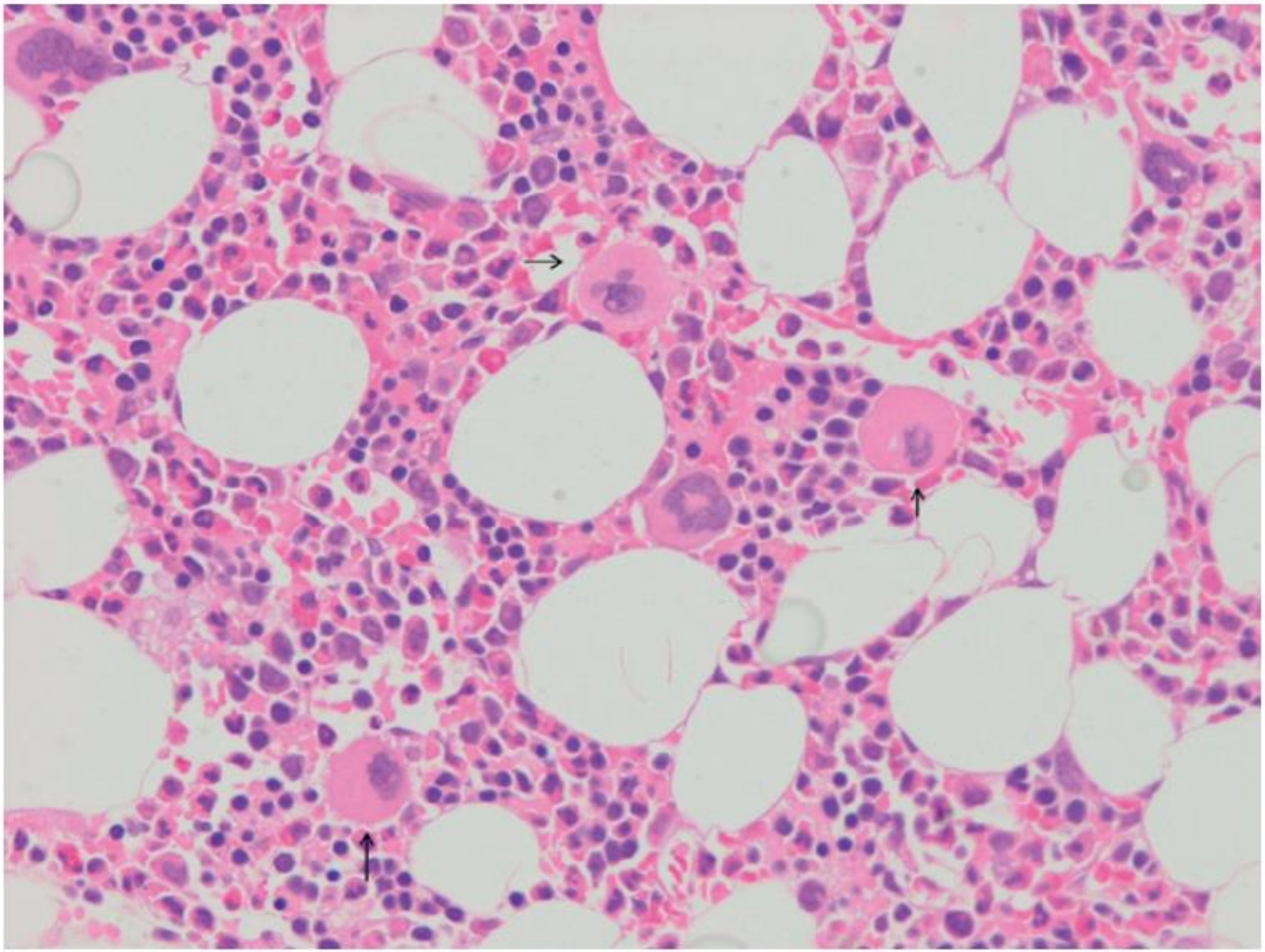
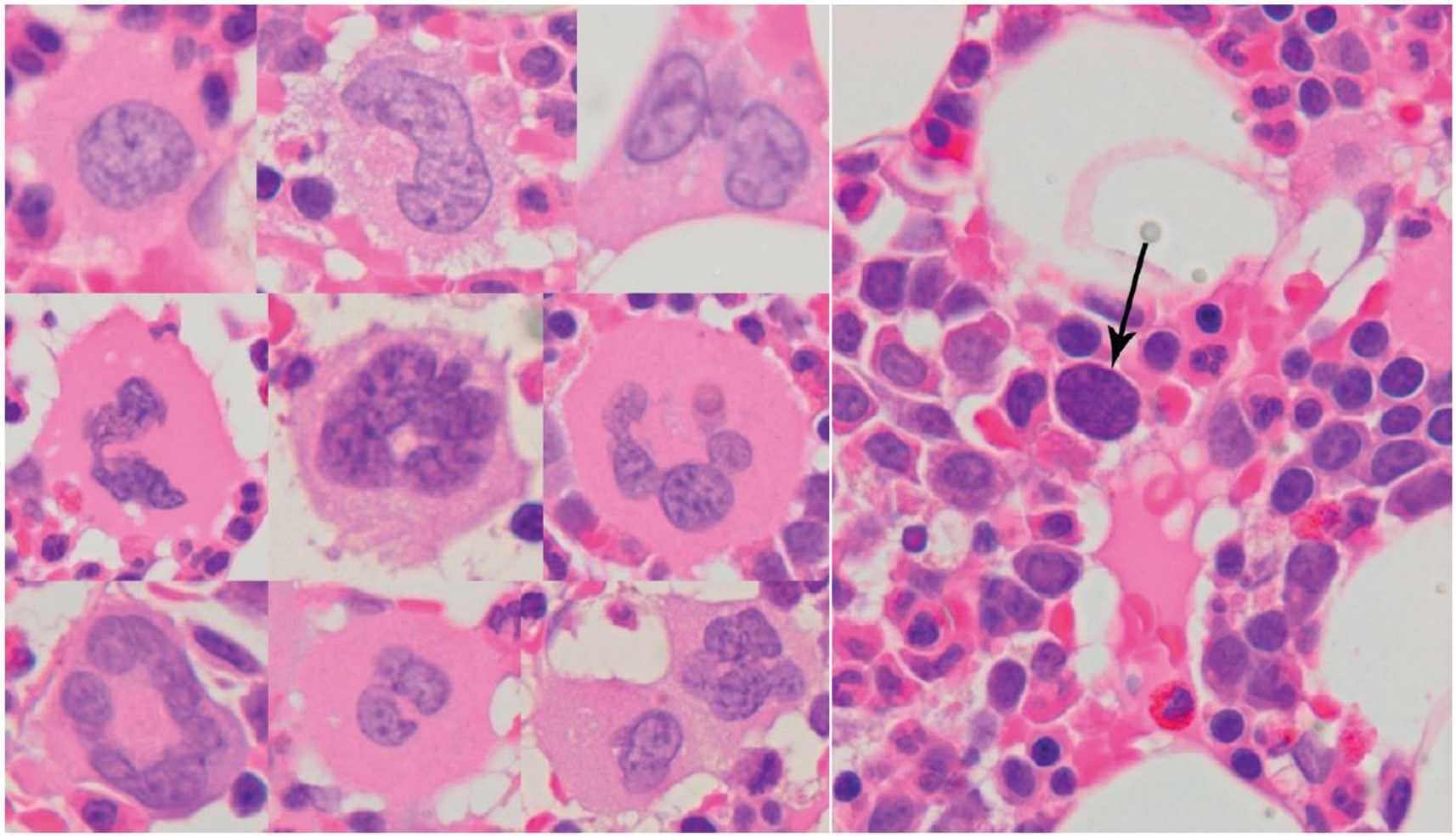


Fig. 3.11 Megakaryocytes, singly scattered and located perisinusoidally. Three cells are seen in close apposition to the sinusoidal wall, extending part of their cytoplasm into the lumen. ×100.



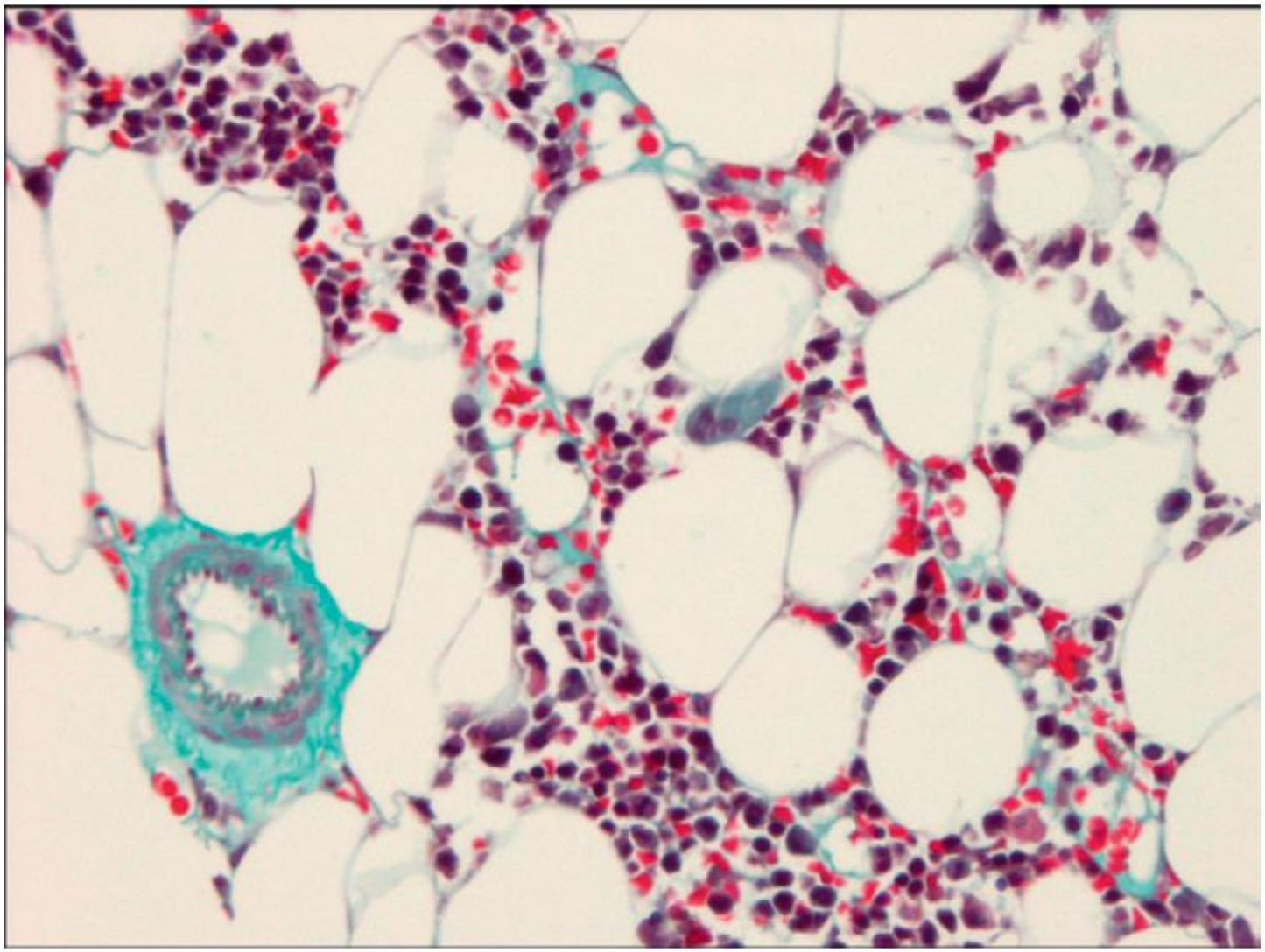


Fig. 3.21 Collagen fibers (green) seen around a blood vessel. Note the lack of collagen between the hemopoetic cells. Masson's trichrome stain. ×200.

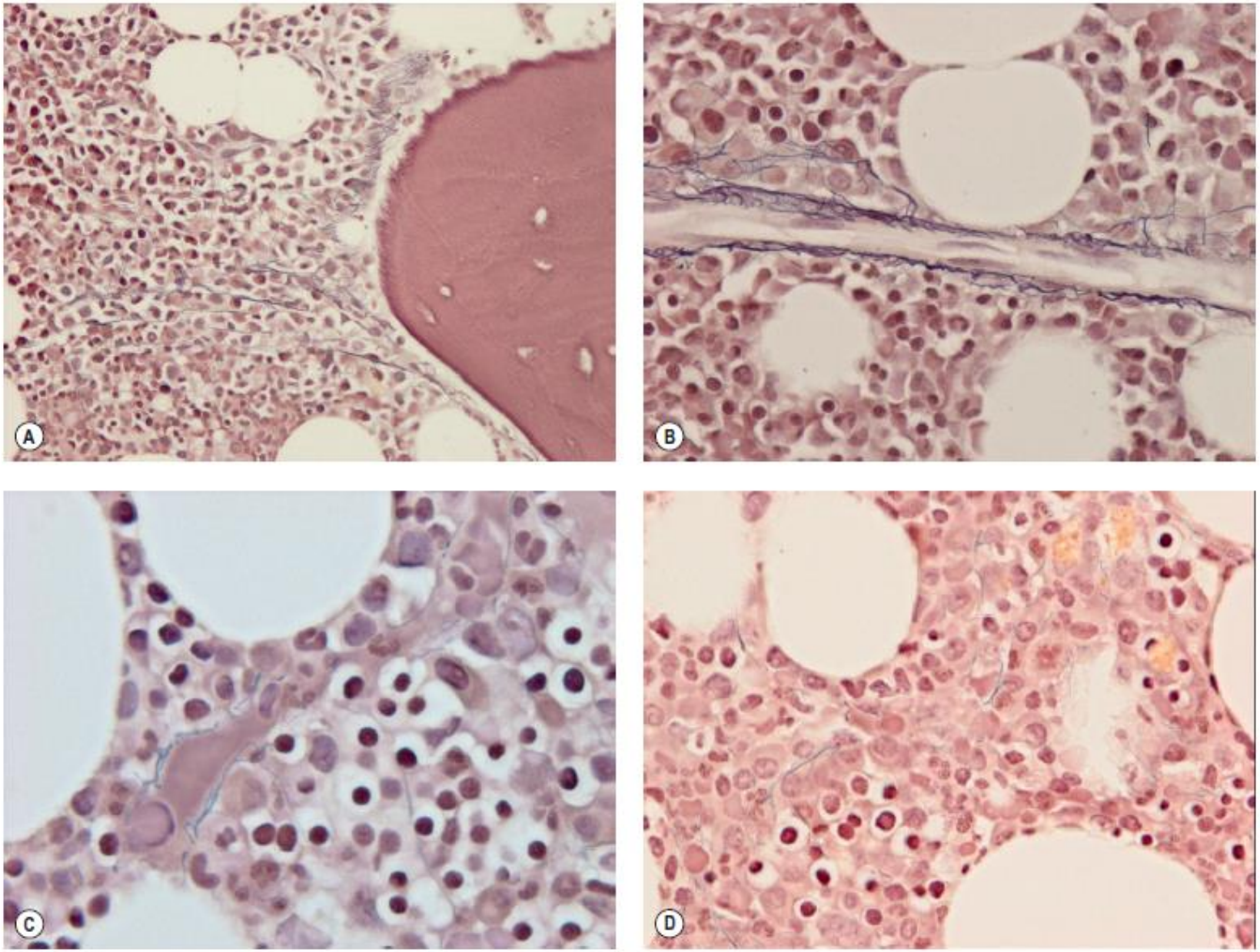
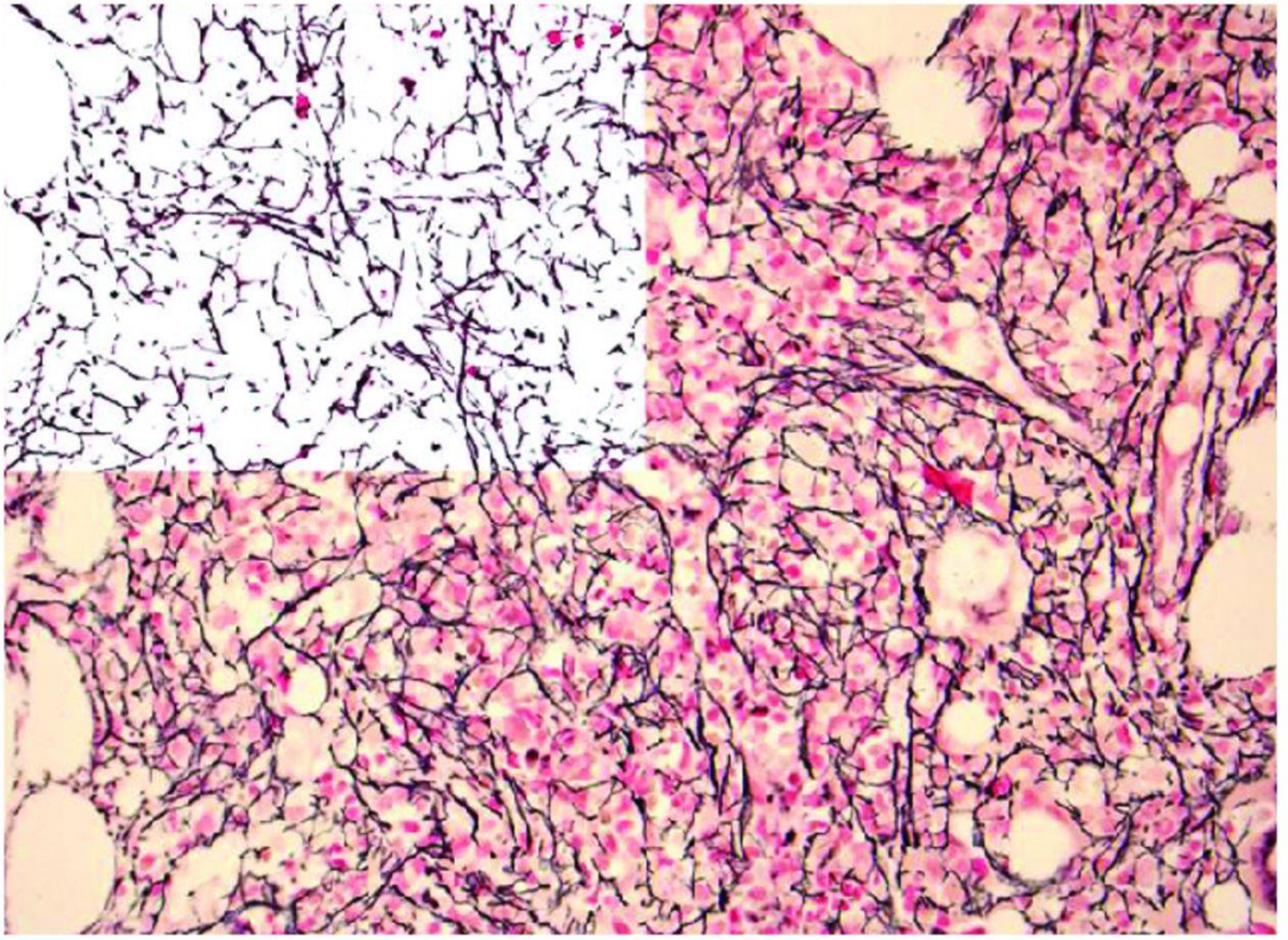


Fig. 3.20 (A) Reticulin attaching/originating from trabecular bone. Gomori stain. (B) Reticulin around a blood vessel. (C) Reticulin forming the wall of a sinusoid. ×400. (D) Typical distribution and density of reticulin in a normal adult marrow. ×200.

Myelofibrosis



Neutrophil alphabet