Flow Cytometry in Transfusion Medicine

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Flow cytometry in the clinical laboratory

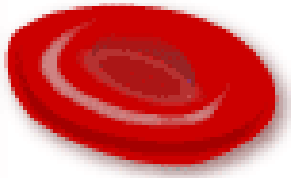
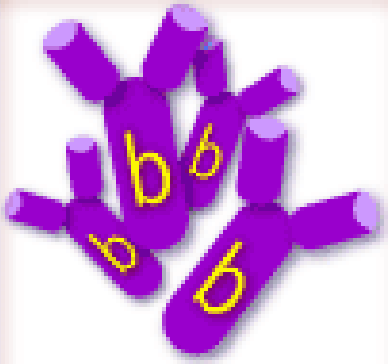
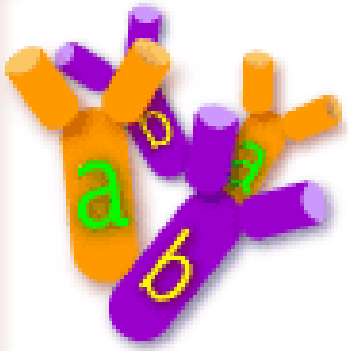
- Lymphocyte immunophenotyping in HIV infection
- Immunophenotyping of leukemias and lymphomas
- Enumeration of CD34+ stem cells
- DNA ploidy and content analysis
- Paroxysmal Nocturnal Hemoglobinuria

Non traditional applications

- Red blood cell antigen and antibody detection (pre-transfusion testing)
- Detection of feto-maternal hemorrhage

Pre-transfusion testing

The ABO Blood System

Blood Type (genotype)	Type A (AA, AO)	Type B (BB, BO)	Type AB (AB)	Type O (OO)
Red Blood Cell Surface Proteins (phenotype)	 A agglutinogens only	 B agglutinogens only	 A and B agglutinogens	 No agglutinogens
Plasma Antibodies (phenotype)	 b agglutinin only	 a agglutinin only	NONE. No agglutinin	 a and b agglutinin

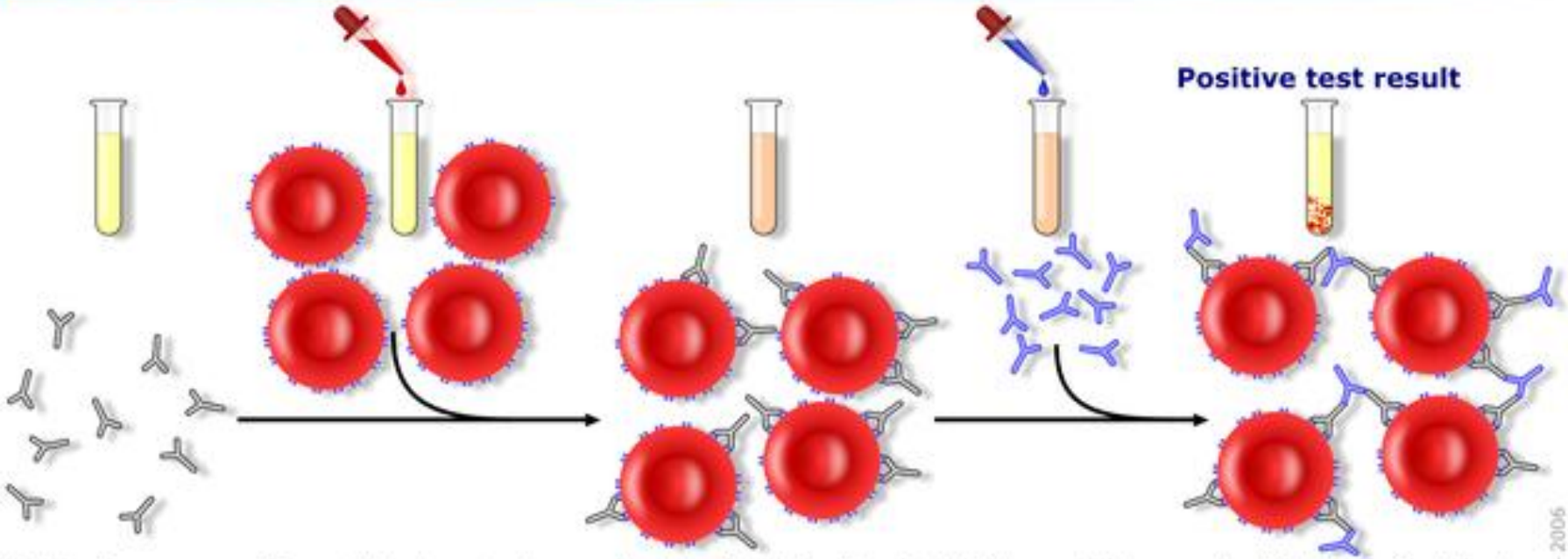
ABO Typing

- Forward Type
  - Add patient cells to known antisera (anti-A, Anti-B)
    - Determines antigens present on RBCs
- Reverse Type
  - Add patient serum to known cells (A cells, B cells)
    - Determines antibodies present in serum

Pre-transfusion Testing

- Type and Screen
  - Determine ABO & Rh (D) type
  - Screen for clinically significant alloantibodies
    - Rh blood group – Anti-C, anti-c, anti-E, anti-e
    - Duffy blood group – Anti-Fy(a), anti-Fy(b)
    - Kidd blood group – Anti-Jk(a), anti-Jk(b)
    - Kell blood group – Anti-K, anti-k
    - Mix 2-3 cell types (known antigens) with patient plasma

Indirect Coombs test / Indirect antiglobulin test



Recipient's serum is obtained, containing antibodies (Ig's).

Donor's blood sample is added to the tube with serum.

Recipient's Ig's that target the donor's red blood cells form antibody-antigen complexes.

Anti-human Ig's (*Coombs antibodies*) are added to the solution.

Agglutination of red blood cells occurs, because human Ig's are attached to red blood cells.

Gel Technology

Problems in Pre-transfusion Testing

- Tube Testing
  - Labor intensive
  - Not amenable to automation
  - Results operator dependent
- Gel Testing
  - Higher costs
  - Slower…not good for emergency testing
  - May not detect all weak antibody-antigen interactions

Pre-transfusion testing via Flow

- Compatibility testing
  - ABO blood group and D typing
  - Antibody screen
  - Identification of alloantibodies

First Hurdle in the application of Flow Cytometry to transfusion medicine

Red cell agglutination is a big problem…

Solutions

1. **Mechanical**
   - Vigorous pipetting and vortexing
   - Small bore needle
2. **Chemical treatment**
   - Glutaraldehyde, formaldehyde, dimethylsuberimidate
3. **Use secondary antibody that does not cause agglutination**
4. **Filter plate technique**
5. **Low speed centrifugation**

ABO and D Typing

- Study by Roback et al.
- Compared ABO typing by flow cytometry with tube method and column agglutination technology (gel)
  - 222 random patient samples

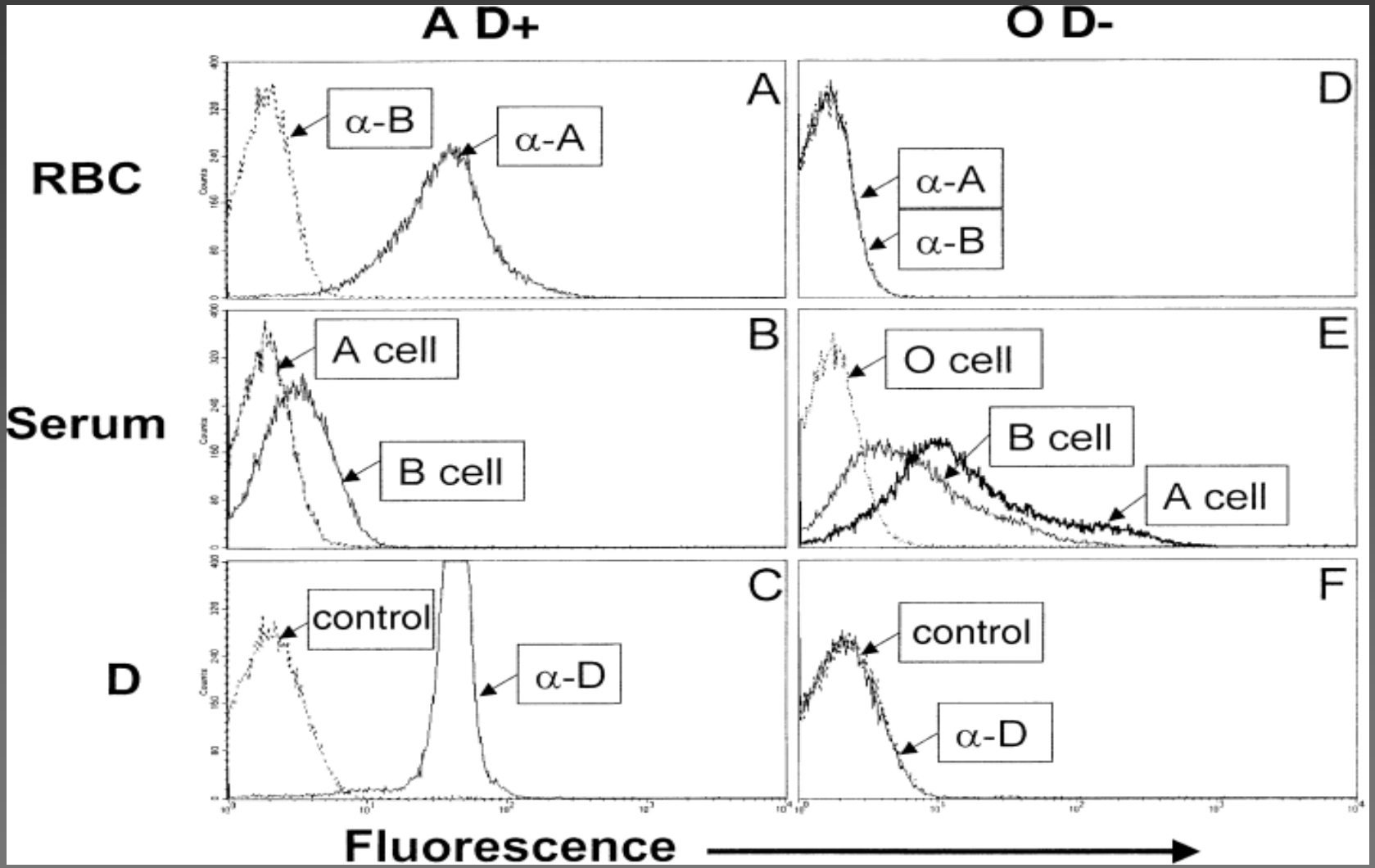
ABO and D Typing

TABLE 1. FC immunohematology protocols for tube and filter plate tests

	Assay			
Step	RBC A,B	RBC Rh(D)	Serum α-A,B	Alloantibodies
1. Add RBCs	2% patient RBCs (25 µL)	2% patient RBCs (25 µL)	3% A, B, O RBCs (30 µL)	3% screening RBCs (25 µL)
2. Add primary Ab	Mse α-A or α-B (50 µL)	FITC Hum α-D (50 µL)	Patient plasma (50 µL)	Patient plasma (50 µL)
3. Add potentiator	NA	20% PEG (100 µL)	NA	20% PEG (100 µL)
4. Incubate	RT × 2 min	37°C × 5 min	NA	37°C × 5 min
5. Wash	Saline × 4 (200 µL)	Saline × 4 (200 µL)	Saline × 4 (200 µL)	Saline × 4 (200 µL)
6. Add secondary Ab	PE-α-Mse IgM (100 µL)	NA	PE-α-Hum IgM (100 µL)	PE-α-Hum IgG (100 µL)
7. Incubate	RT × 5 min	NA	RT × 5 min	RT × 5 min
8. Wash	Saline × 2 (200 µL)	NA	Saline × 2 (200 µL)	Saline × 2 (200 µL)
9. Disperse RBCs	Vigorous pipetting and vortexing (as necessary)			

Roback, et al. An automatable format for accurate immunohematology testing by flow cytometry. Transfusion43(7),918-927.

ABO and D Typing



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ABO and D Typing; Results

- Accuracy
  - FC - 99.1%
  - Tube - 95%
  - Gel - 91.9%
- Conclusions
  - FC performed as well as tube & gel for ABO & D typing
  - FC performed better for some samples
    - Rouleaux
    - Autoantibodies
    - Weak alloantibodies
    - Mixed-field reactions

Alloantibody Detection

239 random patient samples

188 with alloantibodies

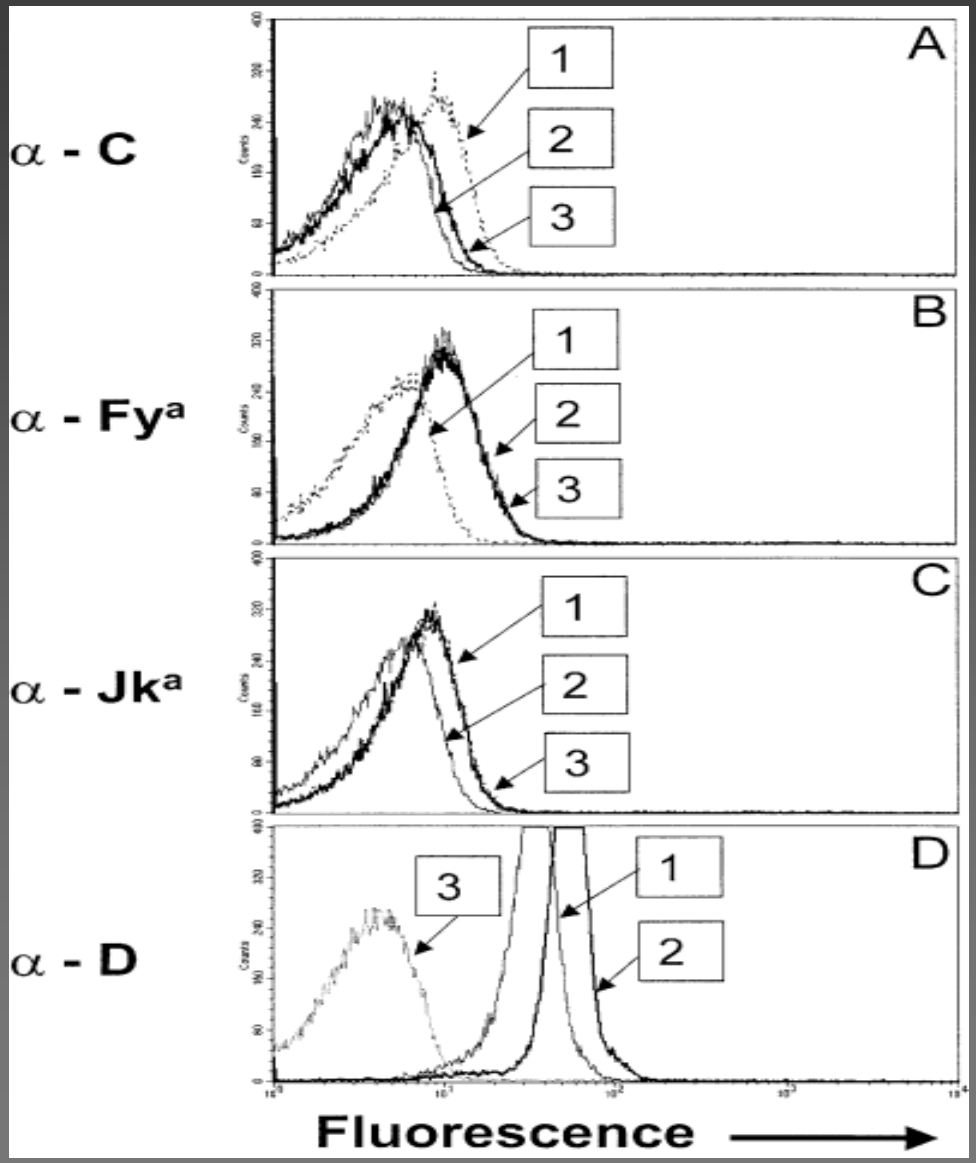
51 with no alloantibodies

Accuracy:

FC - 99.5%

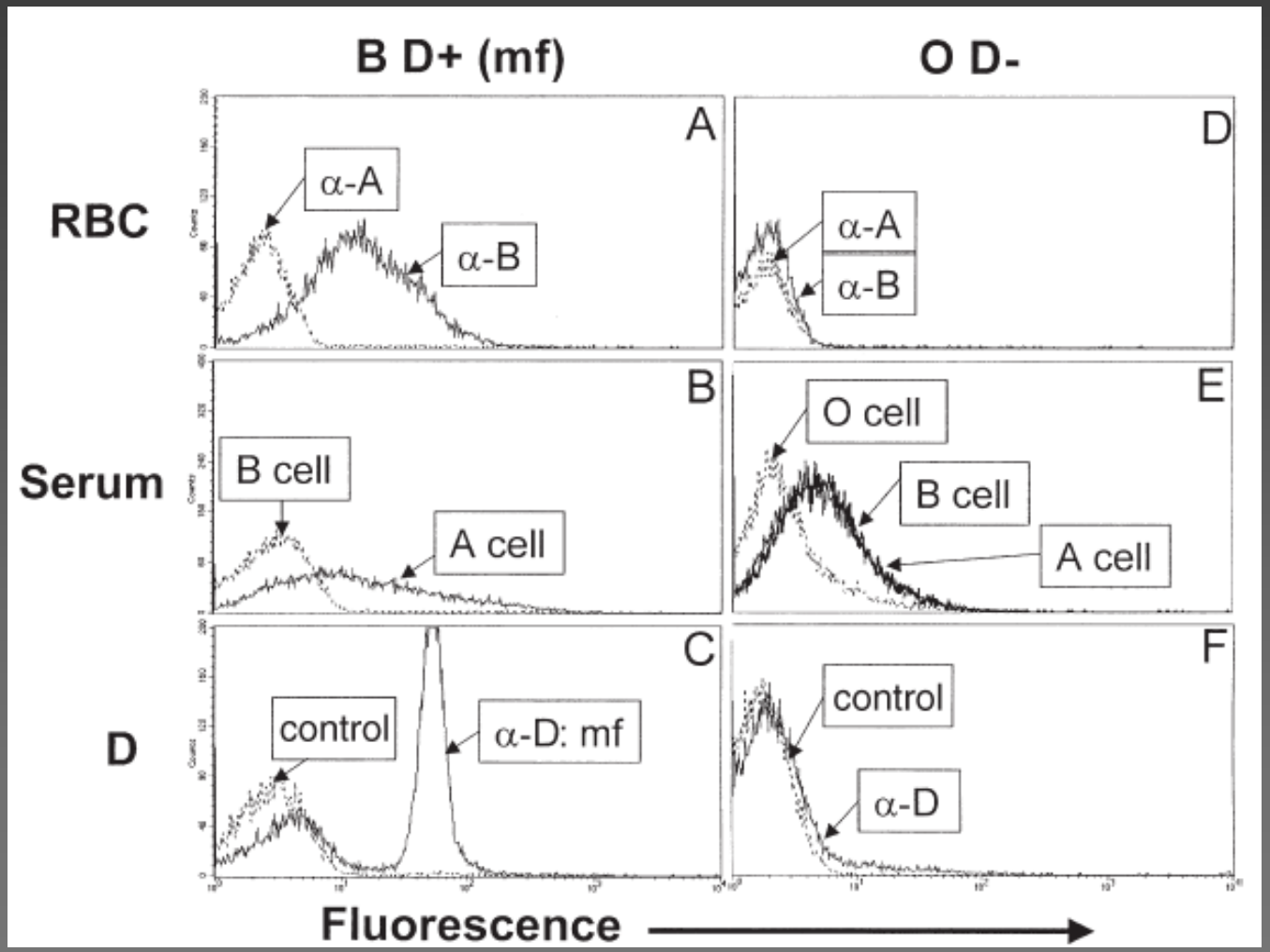
Tube - 94.7%

Gel - 98.9%



From: Roback, et al. An automatable format for accurate immunohematology testing by flow cytometry. Transfusion43(7),918-927.

Subpopulation Detection



Roback, et al. An automatable format for accurate immunohematology testing by flow cytometry. Transfusion43(7),918-927.

Further Study

- ***Roback, et al. Improved method for fluorescence cytometric immunohematology testing. Transfusion 44, Feb 2004; 187-196***
- Personal Cell Analyzer (PCA-96)
  - A capillary cytometry system which can automatically acquire samples in a 96- well plate format
  - Low speed centrifugation used to separate RBCs

Results

- ABO & D Typing Accuracy
  - PCA-96 - 98.7% (229 samples)
  - Gel – 97.4%
- Alloantibody Screens
  - PCA-96 – 99.1% (213 samples)
  - Gel – 99.5%
  - FC was superior in detecting mixed-field reactions ($p < 0.005$)

Results, cont.

- FC showed good well-to-well and day-to-day reproducibility
- FC can detect alloantibodies with both homozygous & heterozygous target RBCs

Flow cytometry is an accurate and sensitive method of pre-transfusion testing and is a promising technique for fully automated testing in the blood bank.

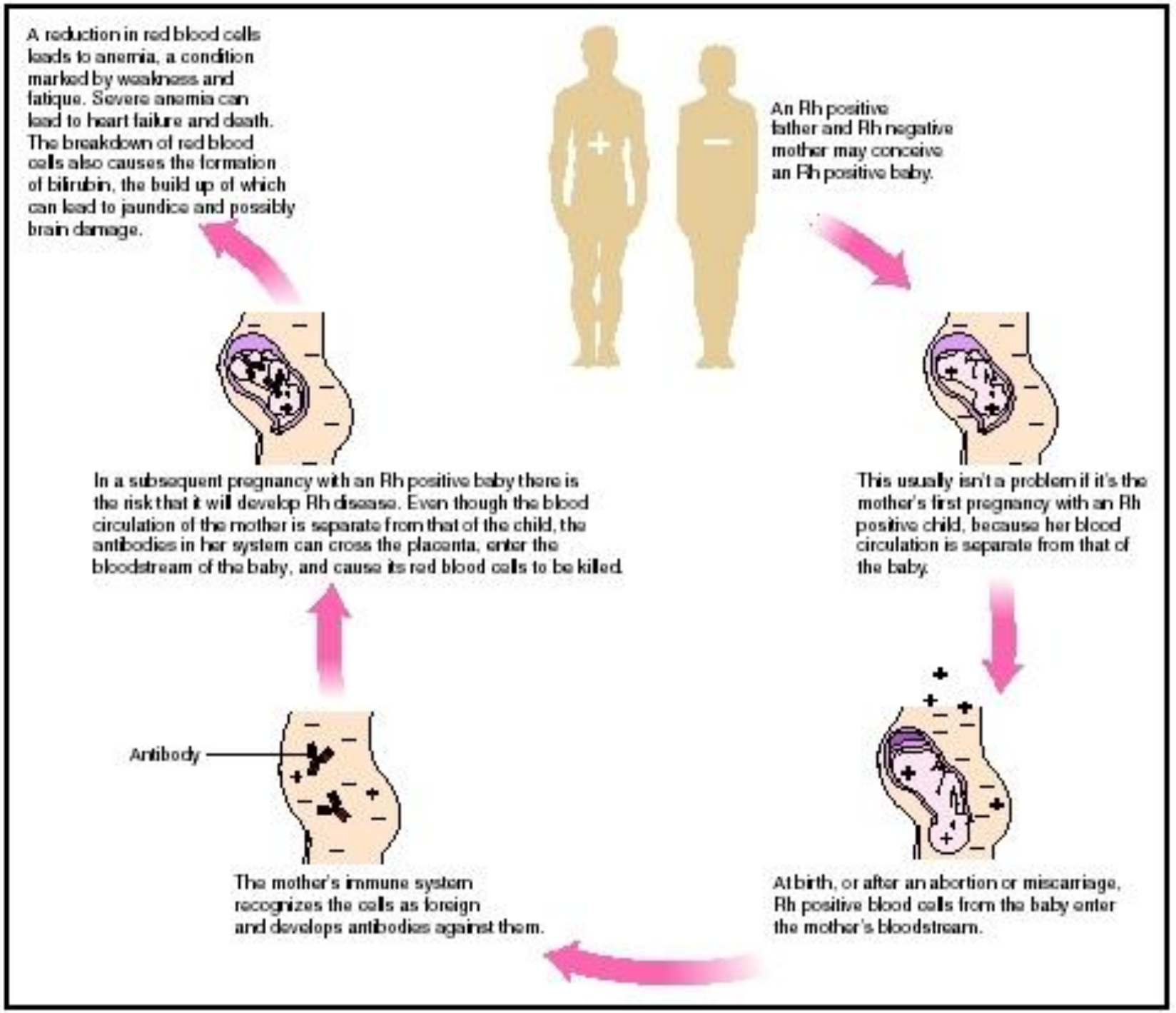
Use of Flow Cytometry to Measure Feto-maternal Hemorrhage

Hemolytic Disease of the Newborn

- **Erythroblastosis fetalis**
- Alloimmune condition that develops in a fetus
  - IgG antibodies produced by the mother (antigen negative) cross the placenta and attack fetal red blood cells (antigen positive)
    - Usually due to anti-D antibodies
    - Can be caused by any red cell alloantibody (anti-C, anti-E, anti-K)
  - The red cells are hemolyzed and the fetus can develop reticulocytosis and anemia
  - Fetal disease ranges from mild to very severe
    - Fetal death from heart failure (hydrops fetalis) can occur

Hemolytic Disease of the Newborn

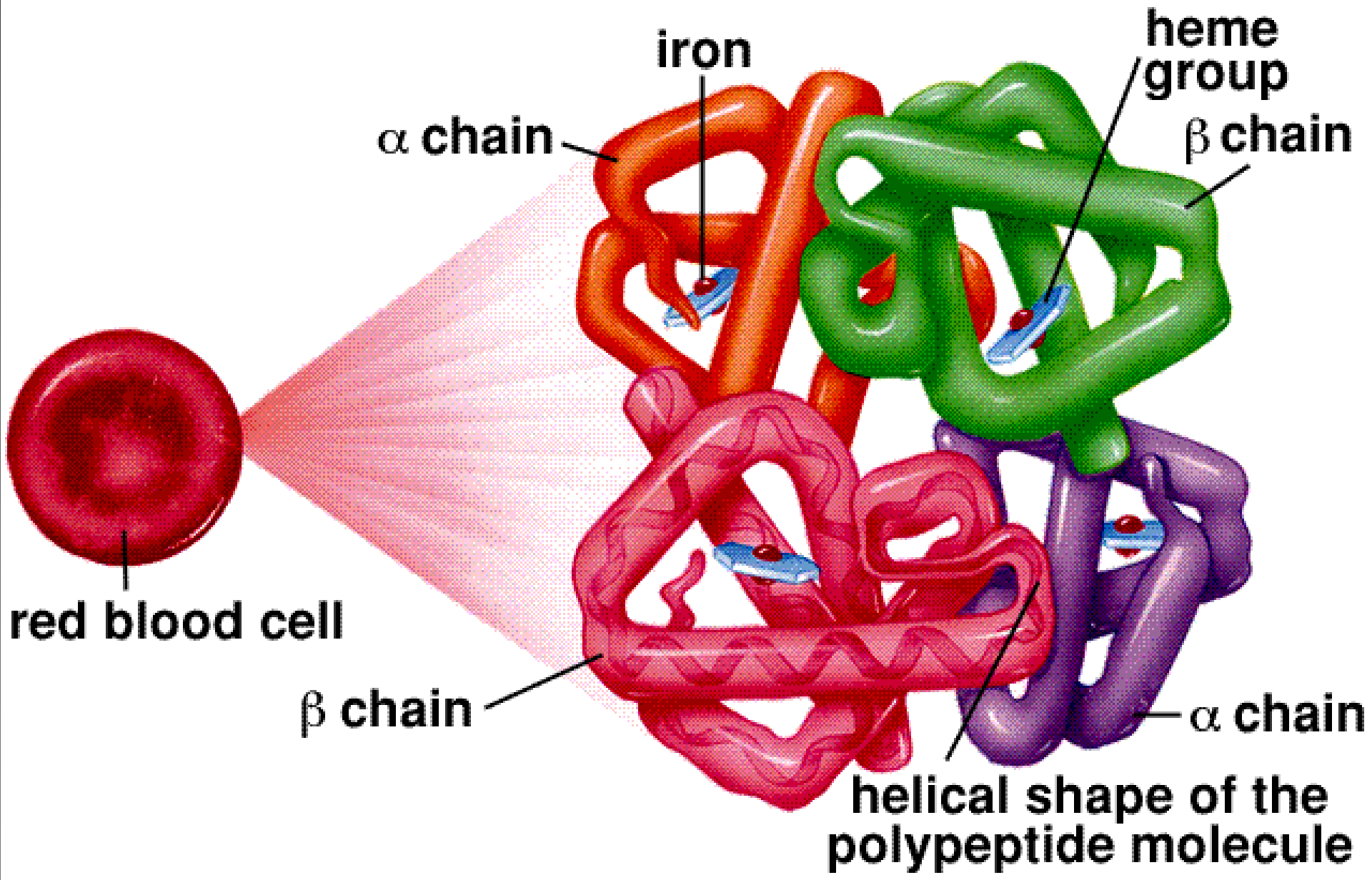
- Alloimmunization
  - Mother forms antibody to antigen she is missing
    - Prior transfusion
    - Prior pregnancy*
      - Mother exposed to fetal blood during delivery
      - Mother exposed to fetal blood as a result of trauma (MVA)

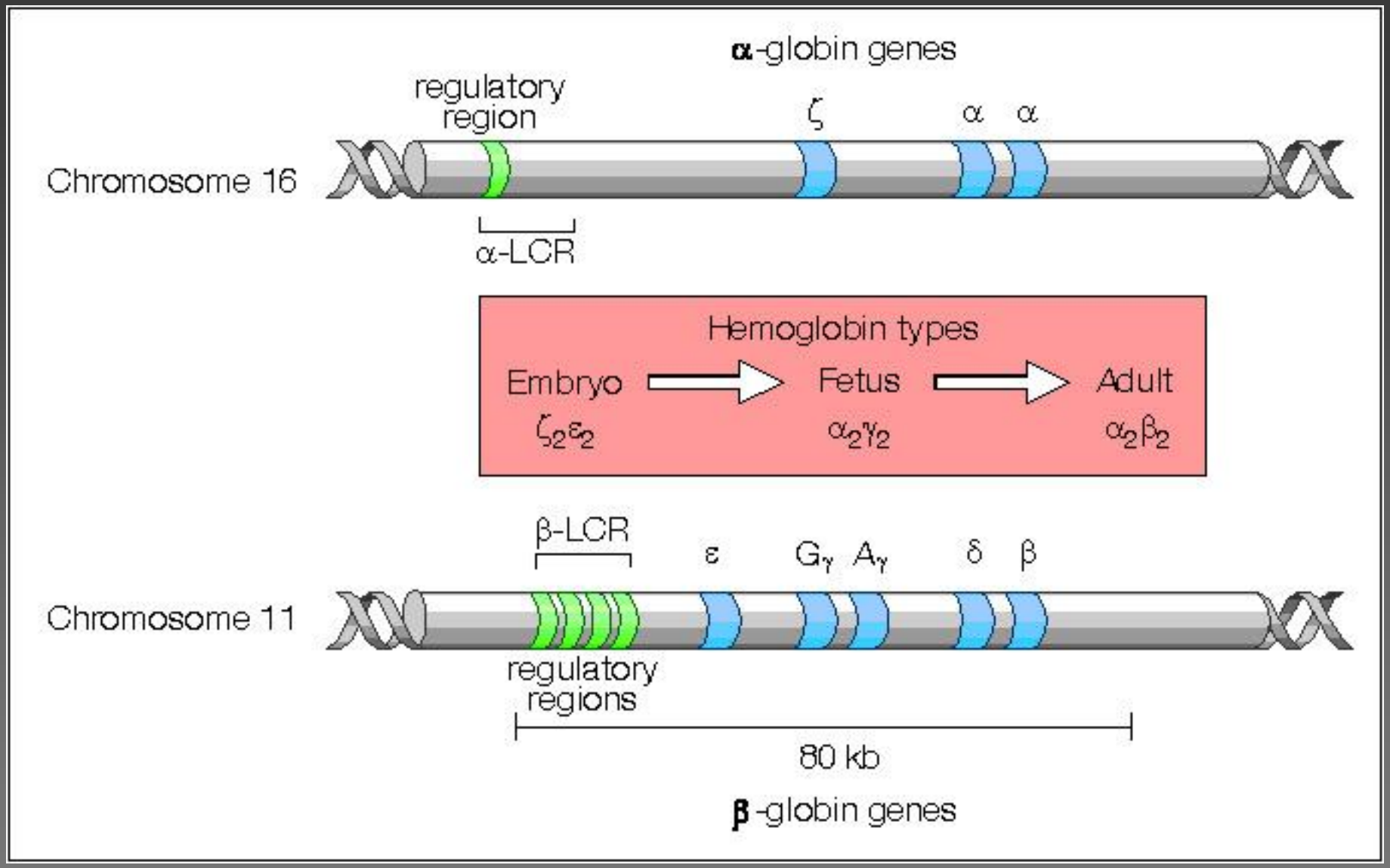

A reduction in red blood cells leads to anemia, a condition marked by weakness and fatique. Severe anemia can lead to heart failure and death. The breakdown of red blood cells also causes the formation of bilirubin, the build up of which can lead to jaundice and possibly brain damage.
An Rh positive father and Rh negative mother may conceive an Rh positive baby.
This usually isn't a problem if it's the mother's first pregnancy with an Rh positive child, because her blood circulation is separate from that of the baby.
At birth, or after an abortion or miscarriage, Rh positive blood cells from the baby enter the mother's bloodstream.
The mother's immune system recognizes the cells as foreign and develops antibodies against them.
Antibody
In a subsequent pregnancy with an Rh positive baby there is the risk that it will develop Rh disease. Even though the blood circulation of the mother is separate from that of the child, the antibodies in her system can cross the placenta, enter the bloodstream of the baby, and cause its red blood cells to be killed.

How can we determine if a mother has been exposed to fetal blood?

Answer: Mother's RBCs contain different hemoglobin than baby's RBCs

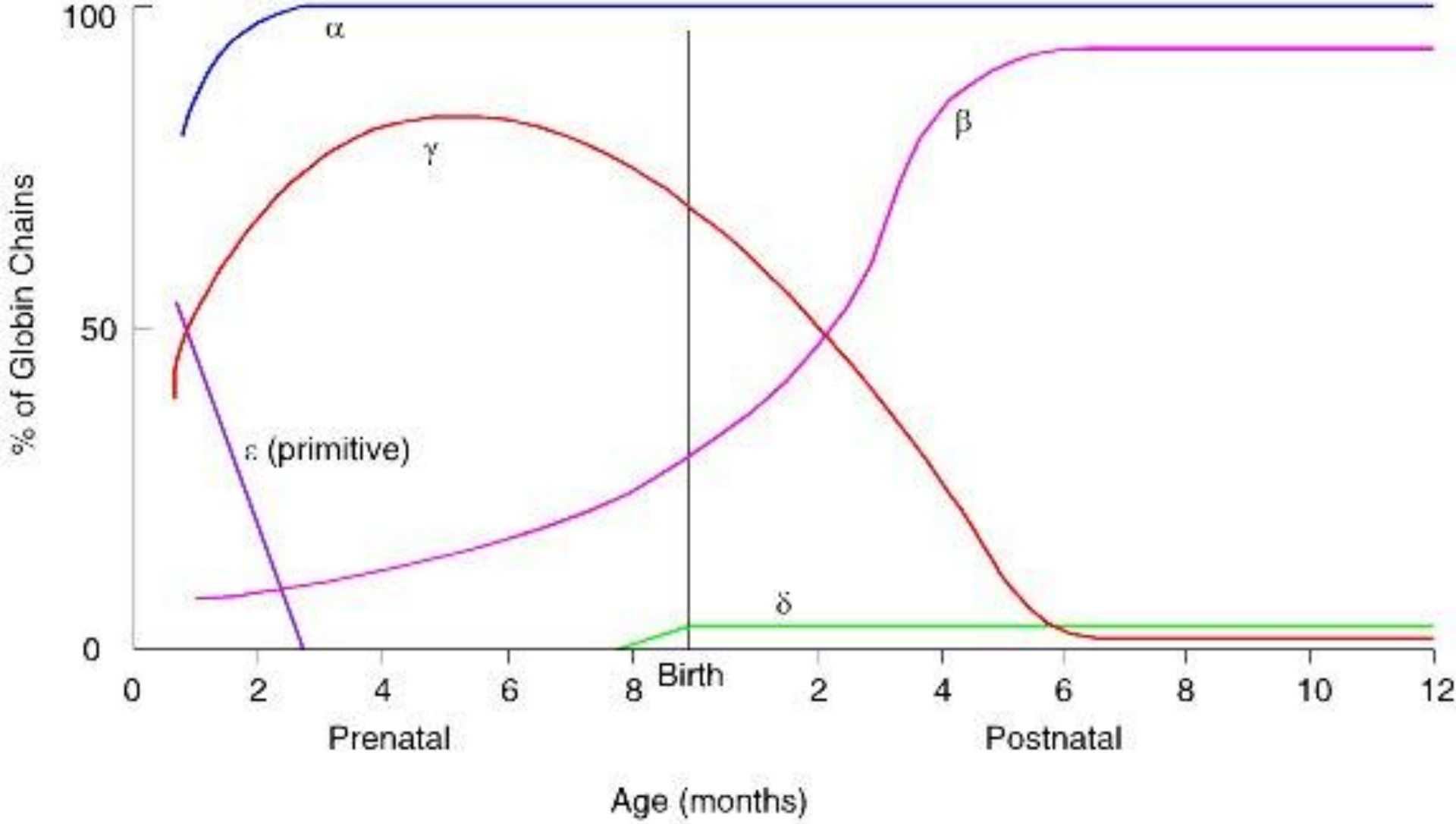
Hemoglobin Molecule




α-globin genes
regulatory region
ζ
α
α
Chromosome 16
α-LCR
Hemoglobin types
Embryo
$\zeta_2\varepsilon_2$
Fetus
$\alpha_2\gamma_2$
Adult
$\alpha_2\beta_2$
β-LCR
ε
G_γ
A_γ
δ
β
Chromosome 11
regulatory regions
80 kb
β-globin genes

Hemoglobin Review

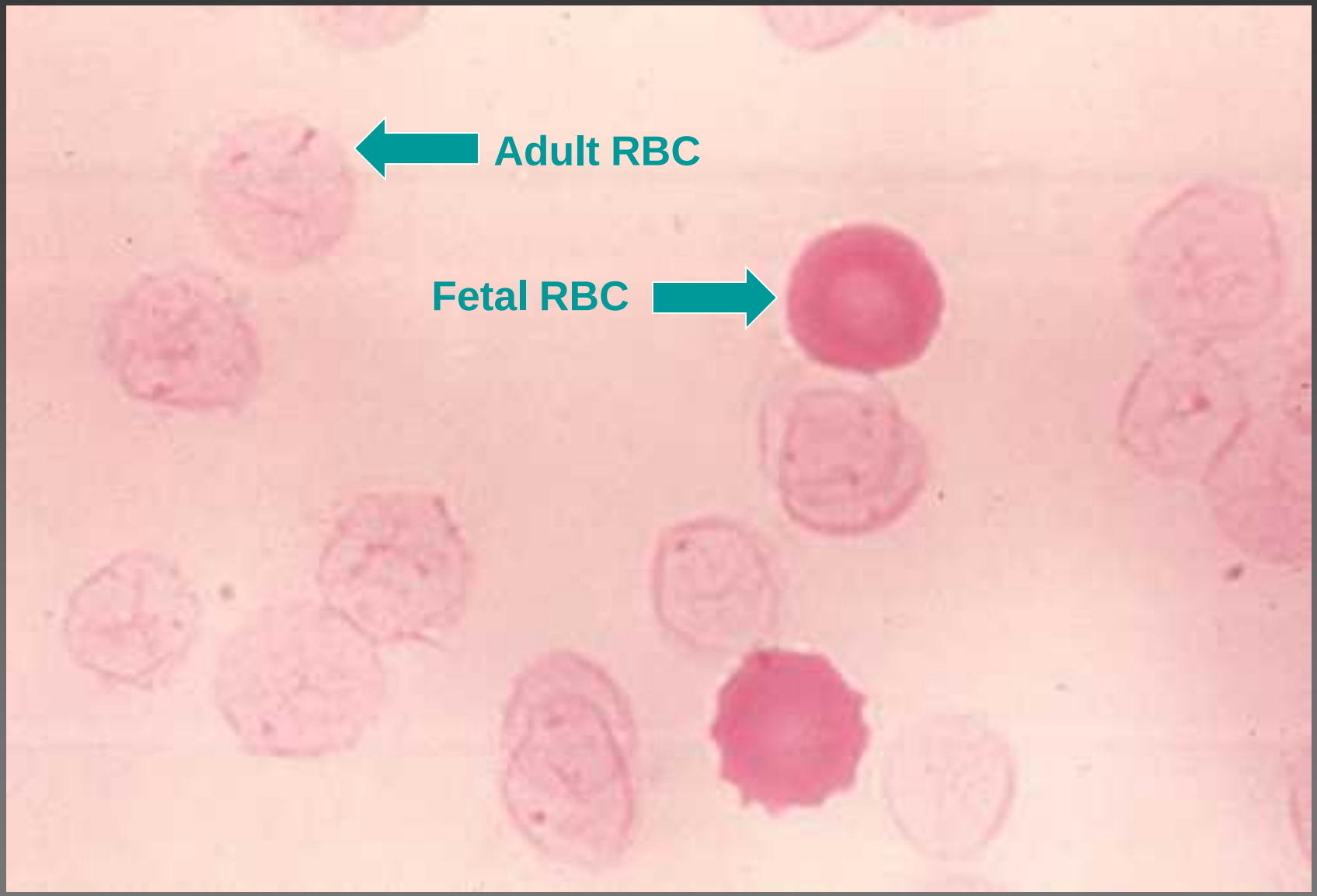
- HbA - >90% of total adult Hb
  - α2β2
- HbA_2 - <3.5% of total adult Hb
  - α2δ2
- HbF – 1% of total adult Hb; 80-100% of total fetal/newborn Hb
  - $\alpha_2\gamma_2$



Relative amounts of the several globin chains (ε, α, γ, β, and δ) present during fetal development and the first year of life.

Identification of Feto-maternal Hemorrhage

- Kleihauer-Betke Test
  - Cytochemical test
  - RBCs containing fetal hemoglobin (HbF) are resistant to acid elution
    - Peripheral smear stained with acid hematoxylin
    - Washed with citrate phosphate buffer


Adult RBC
Fetal RBC

Kleihauer-Betke Test

- Used to quantify amount of Rh immune globulin (RhoGAM) needed to prevent Rh alloimmunization
- Problematic
  - Subjective
  - Imprecise
  - Operator dependent
  - Cannot distinguish maternal cells containing HbF and fetal cells

Identification of Feto-maternal hemorrhage by Flow Cytometry

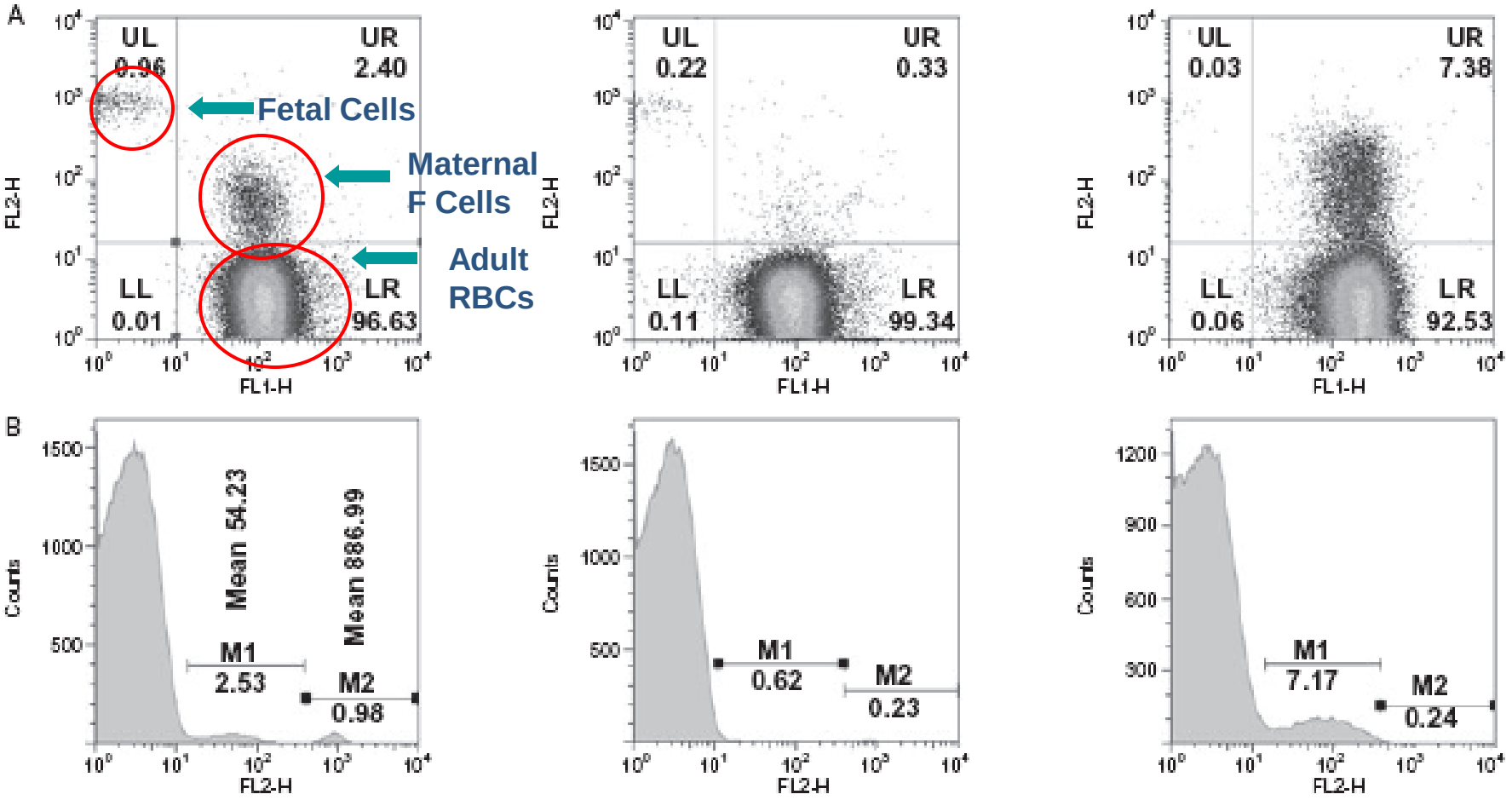
- Quantification of fetal cells identified by specific blood group antigen
  - Anti-D antibodies
  - Antibodies to HbF
    - Some HbF is expressed in all adults
    - Slightly lower HbF expression in adult cells vs. fetal cells
  - Fetal Cell Count Kit

Fetal Cell Count Kit

- Mouse monoclonal anti-HbF
- Polyclonal antibody to carbonic anhydrase
  - Enzyme only fully expressed in RBCs after birth
    - Adult levels at 6 months to 1 year of age
- Allows discrimination & quantification of 3 cell populations
  - Adult RBCs (HbF - , CA +)
  - Fetal RBCs (HbF +, CA -)
  - Adult F cells (HbF +, CA +)

Evaluation of Fetal Cell Count Kit

- Porra, et al. Identification and quantification of fetal red blood cells in maternal blood by a dual-color flow cytometric method: evaluation of the Fetal Cell Count Kit. Transfusion 2007; 47: 1281-1289.
  - Evaluated 455 pregnant or post-partum women & 124 artificial mixtures of adult and 0.01-5% fetal RBCs
    - Compared results of Kleihauer-Betke Test and Flow Cytometric Analysis (Fetal Cell Count Kit)



FL-1 = Carbonic Anhydrase-FITC

FL-2 = HbF-PE

TABLE 2. Comparison of Fetal Cell Count kit versus KBT in patients

	Fetal Cell Count kit		
KB test	Positive	Negative	Total
Positive	44	6	50
Negative	0	405	405
Total	44	411	455

Clinical Application

- RhoGAM injection (300μL) prevents alloimmunization due to 30 mL feto-maternal hemorrhage (0.03% fetal RBCs)
- Quantification of hemorrhage by Fetal Cell Count Kit has detection limit of 0.02% (200,000 cells gated) or 0.03% (100,000 cells gated)

Conclusions of Study

- Fetal Cell Count Kit is more precise than Kleihauer-Betke test for quantification of feto-maternal hemorrhage
  - Greater number of cells counted in shorter period
  - More objective
  - KBT can overestimate number of fetal cells
    - Increased amounts adult HbF cells
    - Erroneous low count of maternal cells
    - Very technician dependent!
  - KBT underestimates sizes of large feto-maternal bleeds

Other applications for flow in transfusion medicine

- Quantitation of residual WBCs after filtration of blood products
- Detection and quantification of RBC-bound IgG, IgM & complement (DAT)
  - Diagnosis of autoimmune hemolytic anemia
- Detection of antibody bound to platelets
- Determination of RBC survival after transfusion
- Phenotyping of recipient RBCs after transfusion
- Detection and quantification of minor RBC populations
  - McLeod carriers
  - Monitoring of bone marrow transplantation engraftment
- Quantification of RBC blood group antigen density