Romanowsky-Giemsa staining

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The Romanowsky-type stain variants such as those originally described or modified by numerous authors, who laid the foundation for modern hematology (the famous EHRLICH, CHENZINSKY, PLEHN, MALACHOWSKI, ROMANOWSKY, NOCHT, JENNER, MAY, GRÜNWALD, LEISHMAN, WRIGHT, GIEMSA etc.), are routinely used for the staining of blood and bone marrow films. Today, we know that a two-component stain is required to produce the so-called Romanowsky-Giemsa effect. Hence, all these stains contain a mixture of methylene blue and related thiazin dyes and eosin. The thiazin component must be azure B according to G GIEMSA (GIEMSA G, 1902, 1903, 1904), A PAPPENHEIM (PAPPENHEIM A, 1901, 1908) and RD LILLIE (LILLIE RD, 1944, 1978 and LILLIE RD et al., 1978), and the eosin component may be eosin Y or one of the corresponding erythrosins. Azure B (methylene azure B) arises by oxidation of methylene blue. Some basics of the Romanowsky-Giemsa stains were studied by PN MARSHALL (MARSHALL PN et al., 1981) and DH WITTEKIND (WITTEKIND DH, 1983).

The typical color of cell nuclei (i.e. purple), is due to molecular interaction between eosin and the azure B-DNA complex. On the molecular nature, however, there exist many theories. The intensity of staining depends on the azure B content and on the ratio azure B / esosin Y. The staining results are influenced by several factors such as fixation, buffer pH, buffer substances and staining time. Minor modifications of working concentrations and staining time were described over the years.

GIEMSA's stain is a member of the ROMANOWSKY group of stains. Because these stains are very tedious to prepare, they are best purchased as pre-made stock stains. The staining methods have been developed for blood films and bone marrow films, but cell smears, cellular imprints, cytospin preparations of different origin and thin tissue sections can be also used. While PAPPENHEIM's staining, i.e. staining by combination of MAY-GRÜNWALD and GIEMSA solutions, is now the standard method in haematology, while GIEMSA staining is the preferred procedure for other histologies. Staining procedures may vary according to the material under study, particularly for tissue sections.

The Romanowsky-type variants lead to some differences in staining. The basic mechanisms and effects, however, are almost the same. Stainings in tissue sections are more variable than in blood films because (and not only due to tissue fixation) tissue sections contain more stainable components. The published protocols report additional steps such as differentiation, dehydration and embedding. Standard procedures do not exist. The whole set-up depends on the cell or tissue type as well as on necessary pretreatments. Hence, experimental evaluation of the method is needed.

Staining must be long enough to develop cellular details. Satisfactory results were reported by use of more dilute staining solutions and longer incubation times. Differentiation is obtained by dilute acetic acid in order to remove excess blue color or by 95% ethanol to remove excess eosin. These steps may be controlled under the microscope.*

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* Dyes and other chemicals in histological staining can be toxic. They must be handled with care

GIEMSA staining of air-dried smears, cytospins, frozen sections

Chemicals	**Chemical solution**
Azure B-Eosin stock solution (Merck), Giemsa's azure eosin methylene blue solution containing Azure B (C.I. 52010) *and* Methylene blue (C.I. 52015) *and* Eosin Y (C.I. 45380) HEPES, free acid HEPES, sodium salt Methanol Distilled water	• HEPES stock A: 0.1 M HEPES (free acid) in distilled water • HEPES stock B: 0.1 M HEPES (sodium salt) in distilled water • HEPES buffer: 900.0 mL HEPES stock A *plus* 100.0 mL HEPES stock B if necessary, adjust pH to 6.5 (buffer is stable for months at 4°C) • HEPES buffer working: 300.0 mL HEPES buffer *plus* 700.0 mL distilled water (corresponds to 0.03 mol/L HEPES) • GIEMSA diluted dye solution: 1.0 mL Azure B-Eosin stock solution *plus* 25.0 mL HEPES buffer working solution

Staining procedure

Air-dried specimens are fixed and stained:

– methanol	3-5 min
– GIEMSA diluted dye solution	5-10 min
– HEPES working buffer	2 x 1 min
– distilled water	1 x rinse

Slides are air-dried in vertical position

GIEMSA staining of paraffin sections

GIEMSA'S stain is widely used for blood and bone marrow films. However, the method is also applied to thin (2-4 µm thick) paraffin sections. Tonsils can serve as control material.

Chemicals	**Chemical solution**
GIEMSA stock solution (Merck), (Azure eosin methylene blue solution) containing Azure B (C.I. 52010) *and* Methylene blue (C.I. 52015) *and* Eosin Y (C.I. 45380) Sodium acetate	• Acetate buffer stock solution: 0.2 M acetate buffers with different pH (in the range of pH 3.5 to 7.0) are prepared and examined in preliminary experiments because pH optimum for the study material should be determined previously) • GIEMSA diluted dye solution:

Glacial acetic acid n-Butanol Distilled water	1.0 mL Giemsa stock solution *plus* 50.0 mL 0.02 M acetate buffer solution (1:10 dilution of 0.2 M acetate buffer, the right pH is adjusted with 5% acetic acid) • Acetate wash buffer: 10.0 mL 0.2 M acetate buffer *plus* 90.0 mL distilled water pH is adjusted with 5% acetic acid • 0.01% acetic acid

Staining procedure

Sections are deparaffinated in the conventional manner, then rehydrated in descending series of ethanol, passed into distilled water and stained:

– GIEMSA diluted dye solution	1-2 hours
– acetate wash buffer	3 dips
– blot dry and control with the microscope; if "blue" predominates, differentiate in acetic acid	
– differentiate in 0.01% acetic acid	optional under microscopic control *
– acetate wash buffer	2-3 dips
– blot dry	
– n-butanol	2 x 3 min

Slides are cleared in xylene or xylene substitute and mounted in resinous medium under coverglass. Always use separate rinse baths

* Fixation influences the color balance of Giemsa staining, and best results are obtained with appropriate pH of the staining solution. Adequate pH is determined by trial with Giemsa staining solutions prepared with acetate buffers in the range from pH 3.5-7.0. If "blue" predominates, then the pH is too high, and if "red" predominates, a higher pH must be chosen

GIEMSA staining of paraffin sections (method according to MERCK)

GIEMSA'S stain may be used as an overview staining method in histology. Sections (2-4 µm thick) of formalin, paraffin embedded tissue serve as material.

Chemicals	**Chemical solution**
GIEMSA azure eosin methylene blue solution for microscopy, concentrated staining solution (Merck, Cat. No. 109204) Glacial acetic acid (100 % anhydrous) Distilled water 2-Propanol Xylene	• Acetic acid 0.1%, aqueous solution: 1 mL glacial acetic acid *plus* 1000 mL distilled water • GIEMSA azure eosin methylene blue solution, undiluted and filtrated

Staining procedure

Sections are passed through xylene and ethanol baths into distilled water, then stained:

– Distilled water	10 sec
– GIEMSA dye solution (undiluted, filtrated)	15 min *
– acetic acid 0.1 %	10 sec
– distilled water	10 sec
– 2-Propanol	10 sec
– 2-Propanol	10 sec
– 2-Propanol	10 sec
– Xylene	5 min
– Xylene	5 min

The stain remains stable when mounted with non-aqueous mounting media (e.g. Entellan). Use separate rinse baths. After dehydration and clarification with xylene, slides are mounted in resinous medium under coverglass

* The resulting stain can be influenced by fixation, staining times, pH value of the incubation solutions

PAPPENHEIM staining (May-Grünwald-Giemsa according to MERCK)

MAY-GRÜNWALD'S stain is frequently used in combination with other dye solutions such as GIEMSA'S solution in hematology and histology, the so-called PAPPENHEIM staining (MAY-GRÜNWALD-GIEMSA, MGG). The combination of both dyes is very popular for staining air-dried smears from body fluids and tissues, for thin preparations such as blood films, cytocentrifuge preparations and other cytological probes (f.e. lymph node aspirates). From the numerous modifications, the MERCK procedure is as follows.

Chemicals	**Chemical solution**
MAY-GRÜNWALD eosine-methylene blue solution for microscopy (Merck, Cat. No. 101424) GIEMSA azure eosin methylene blue solution for microscopy (Merck, Cat. No. 109204) Phosphate buffer tablets pH 6.8 for preparing buffer according to WEISE (Merck, Cat. No. 111374) Distilled water	• Working buffer solution pH 6.8: 1 buffer tablet *plus* 1000 mL distilled water • MAY-GRÜNWALD staining solution: 30 mL stock solution *plus* 20 mL buffer solution pH 6.8 *plus* 150 mL distilled water • GIEMSA staining solution: 10 mL stock solution *plus* 190 mL buffer solution pH 6.8

Staining procedure on the staining rack

Air-dried specimens, slides must be completely covered, slides are allowed to drip off well after each staining step to avoid unnecessary cross-contamination of solutions:

– MAY-GRÜNWALD staining solution	3 min

– buffer solution	add 1 mL buffer solution
– GIEMSA staining solution	20 min
– buffer solution	rinse
– air-dry	overnight
– covering with non-aqueous mounting media	
The stain remains stable when mounted with non-aqueous mounting media (e.g. Entellan). Use separate rinse baths. After dehydration and clarification with xylene, slides are mounted in resinous medium under coverglass	
* The resulting stain can be influenced by fixation, staining times, pH value of the incubation solutions	

PAPPENHEIM staining (DGHO standard stain according to BINDER T et al. 2012)

The Romanowsky-Giemsa effect depends largely on a sufficient alcoholic fxation. Even then the staining quality is just assured by rigorous control of the whole staining procedure. Standardized protocols are to be preferred for reliable staining results of nucleic and cytoplasmic details. To this aim, the DEUTSCHE GESELLSCHAFT FÜR HÄMATOLOGIE UND ONKOLOGIE (DGHO) published the description of a standard procedure for the PAPPENHEIM stain (BINDER T et al., 2012). They recommend this procedure to obtain inter-laboratory identical results.

Chemicals	**Chemical solution**
Methanol (Merck, Cat. No. 106009) MAY-GRÜNWALD eosine-methylene blue solution for microscopy (Merck, Cat. No. 101424) GIEMSA azure eosin methylene blue solution for microscopy (Merck, Cat. No. 109204) Phosphate buffer tablets pH 6.8 for preparing buffer according to WEISE (Merck, Cat. No. 111374) Distilled water	• Working buffer solution pH 6.8: 1 buffer tablet *plus* 1000 mL distilled water (pH control advised) • MAY-GRÜNWALD staining solution: 60 mL stock solution *plus* 30 mL buffer solution pH 6.8 • GIEMSA staining solution: 10 mL stock solution *plus* 60 mL buffer solution pH 6.8 (filtration is optional, but pH control advised)
Staining procedure in Coplin or Schiefferdecker jars It is important to fix air-dried specimens in methanol prior to staining. Staining solutions are freshly prepared, staining jars are covered by lids:	
– Methanol (absolute)	10 min (at least)
– MAY-GRÜNWALD staining solution (*alcoholic staining*)	7 min
– preparations are dripped off	no washing
– GIEMSA staining solution	20 min

	(*aqueous staining*)	
–	buffer solution	10 sec (short immersion)
–	buffer solution	4 min (*differentiation*)
–	buffer solution	4 min (*differentiation*)
–	air-drying	air drying by ventilation
The stain remains stable when mounted with non-aqueous mounting media (e.g. Entellan). After clarification with xylene, slides are mounted in resinous medium under coverglass		
* The resulting stain can be influenced by fixation, staining times, pH value of the incubation solutions		

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Full citation of publications is given in chapter *References*
link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/12/References.pdf

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