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Romanowsky staining, the Romanowsky effect and thoughts on the question of scientific priority

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Abstract

I give an historical account and analysis of the scientific priority of the discovery of the polychrome staining of microscopic biological preparations provided by mixtures of eosin plus methylene blue and its derivatives, especially azure B. I maintain that both the formal priority for the discovery of the polychrome staining phenomenon and credit for initiating the development of a technique of polychrome staining properly belong to D. L. Romanowsky. His scientific work demonstrated the possibility of using a simple technique to stain hematological preparations selectively to give good contrast, high resolution and the ability to identify malaria parasites. Romanowsky's approach constituted the starting point for the development of a family of polychrome stains for microscopic investigation of hematological preparations by a number of his contemporaries.

Key words: azure, eosin, Malachowski, malaria parasite, methylene blue, Romanowsky, Romanowsky effect, Romanowsky stain

I address here the origins of staining techniques that use eosin plus various methylene blue derivatives, which must include azure B, to give polychrome staining for microscopy of biological preparations. These methods have been widely used in various fields of medicine and biology for more than 100 years. The techniques, stains and characteristic staining outcomes have been described as Romanowsky staining, Romanowsky-type stains and the Romanowsky effect, respectively. Several scientists have referred to this type of stain as the Romanowsky-Malachowski stain (Lillie 1978) and even simply the Malachowski stain (Krafts et al. 2011a,b, Ward et al. 2011). The authors of these articles seem not to have accounted for the findings published in the first Romanowsky article (Romanowsky 1890) and in his dissertation (Romanowsky 1891a), which preceded the Malachowski article (Malachowski 1891). Consequently, it is necessary to analyze systematically the chronology and key contributions concerning the discovery of the stain and its staining outcomes by two scientist-physicians: a Russian, Dmitry Leonidovich Romanowsky, and a German, Ernst Malachowski. A preliminary version of my article was available earlier online (Bezrukov 2010). Readers also may be interested in a portrait of Romanowsky (Fig. 1), which is from the family archives. A corresponding image of Malachowski already has been published (Krafts et al. 2011a).

Preliminary remarks concerning terminology and general topics

Below, the default term for describing the stains under consideration is the traditional one, namely, "Romanowsky stains." There are, however, several definitions of Romanowsky stains and staining, and of the Romanowsky effect. The best known were given by Baker (1958), Horobin (2011), Marshall (1978), Wittekind (1983) and by a group of International Council for Standardization in Haematology (ICSH) experts (Wittekind et al. 1983). These definitions possess both advantages and disadvantages. For example, the definition provided by the group of ICSH experts

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Fig. 1. Photo of D. L. Romanowsky taken in Kislovodsk, a city in southern Russia, sometime between 1910 and 1919. By kind permission of Romanowsky's granddaughter, A. M. Kurco.

does not include certain procedures, such as the Field stain and the various Diff-Quik methods, in which the anionic and cationic dyes are applied separately. Consequently, I shall use a modified definition, more suited to the topic under consideration here. The Romanowsky effect is the polychrome staining of biological preparations after application of stains containing demethylated derivatives of methylene blue (azure B, azure A etc.) plus a red-orange halogenated fluorescein dye (usually eosin Y). After such staining, the characteristic color of cell nuclei and of granules of neutrophil polymorphs is purple with an absorption maximum at approximately 550 nm. In addition, other cell structures can appear red-orange, brick red, pink, red, blue, light blue, black, violet-black, violet-red etc. Romanowsky stain is any stain technique that produces the Romanowsky effect.

The term "Romanowsky-Giemsa effect" is somewhat problematic. Romanowsky discovered the polychrome staining effect, while Giemsa developed a particularly successful stain composition and staining procedure for the Romanowsky stain. The original Giemsa stain was available commercially under the label "Giemsasche Lözung für die Romanowsky Färbung" which translates to "Giemsa solution for Romanowsky staining" (Giemsa 1902, Nikiforov 1919, Fleischer 2004).

The date of a scientific priority in this article is understood as the date of the first documented publication (disclosure to an unrestricted circle of people) of new scientific achievement. It is important also to understand that Romanowsky and Malachowski articles were published at a time when the Julian calendar was still in use in Russia, while Germany was using the more modern Gregorian system. Therefore, I have converted the dates of the Russian publications to the Gregorian system to permit easy comparison.

Key results concerning staining of blood and the malaria protozoan parasite published by Romanowsky and Malachowski during 1890–1891

Romanowsky's publications

In December 1890, D. L. Romanowsky published a "preliminary report" (Romanowsky 1890). In this he wrote: "For staining the following mixture is used, as discovered by me, which is best when freshly prepared: 2 volumes of a filtered saturated aqueous solution of methylene blue plus 5 volumes of a 1% aqueous eosin solution." This report included a detailed description of technique including sample preparation, how the stock and working solutions of the stain were formulated and an account of experimental variants. No details concerning preparation of the methylene blue solution were provided, although in referring to the (eosin-free) methylene blue staining technique devised by two Italian scientists, Celli and Guarneri (1888/1889), Romanowsky emphasized that their solution (methylene blue solution in ascites fluid) was a "non-rotten preparation." Romanowsky cited these researchers by name in his 1890 article, but he did not provide a bibliographic reference; this bibliographic information subsequently appeared in his 1891 thesis, which appears in the list of references (Celli e Guarneri 1888/1889).

Romanowsky described the outcomes of his staining procedure in some detail, as follows:

> In my preparations I always obtain the following picture. Red cells are stained in a pink color. Cytoplasm in eosinophils is saturated-pink, whilst that in the malaria parasite and lymphocytes is light blue. Blood platelets and the nuclei of white cell are dark-violet, whilst the nuclei of malaria parasites are purple-violet. The cytoplasm of leukocytes is pale-violet, with transitional colors between the light blue protoplasm of lymphocytes to violet leukocytes.
>
> Within red cells the malaria parasite may be hardly noticeable or may occupy the whole cell. In any event, the violet nucleus, surrounded by a colorless rim, is always clearly distinguishable. Whether the nucleus is circular or egg-shaped, or indeed a distorted triangular form, probably depends on the condensation of a chromatin network.

The fine structure of the three-day fever parasites at different stages of development also was described in some detail. Finally, Romanowsky concluded the article by writing: "And so, applying the stain described by me, it is possible to demonstrate the presence of a nucleus in malaria parasites in dried blood smears. Such observations were not previously possible using other procedures. I saw a nucleus in a parasite on the same site as did the Italian authors."

A German abstract of this article was prepared by Rothert (1891). Lillie (1978) stated that this source provided no details on staining of blood cells, but did report "red parasite chromatin in a blue plasmodium." A complete English translation of the first Romanowsky article is available on-line (Romanowsky 1890).

A detailed description of stain preparation and techniques was provided later in D. L. Romanowsky's Doctor of Medicine dissertation (Romanowsky 1891a, pp. 63–75). He defended his thesis on May 30, 1891. Based on Ehrlich's concept of "neutral dyes," he used the volumetric ratio of a saturated aqueous solution of methylene blue to a 1% aqueous eosin solution that resulted in precipitation when the solutions were mixed. "In this case, the mixture has great staining ability, nuclei especially being well stained, and the dye does not lose its selective features. Moreover, in addition to the initial dyes, a third dye appears in the mixture, which has a characteristic color, and the greatest affinity to nuclei, or more exactly to their chromatin." Here, Romanowsky considered that there is a third dye in the mixture, or that a third one is generated in situ. As we now know, the third dye was azure B, an oxidation product of methylene blue which, in combination with eosin, stains nuclear chromatin purple (Horobin 2011). From our current understanding, it is clear that "the third dye" was already present in the aged ("moldy") methylene blue solution.

Romanowsky explained further how the solutions and mixtures were prepared. In particular, he emphasized that an aged solution of methylene blue was used:

> … methylene blue starts staining optimally when mould appears on the surface of the solution, looking like a white film. We always have on hand a large bottle containing methylene blue, as a saturated solution, with excess dye present as a sediment. As aliquots of dye solution are removed, we add water, shake up the solution and the sediment and, after settling, filter aliquots prior to use.
>
> … note that the old methylene blue solution requires less eosin for its saturation. So it is necessary to determine the volume ratio required to achieve precipitation every two months. After aging for 9 months, 1 volume of methylene blue no longer requires 2 but 1½ volumes of eosin.

Probably during long storage, methylene blue gradually was oxidized and formed azure B and other lower homologues, leading to polychromatic staining of smears. Romanowsky commented that "our experience demonstrated that dye lots from different companies are not identical," but he did not state precisely which dyes were used or from which firms the dye lots were obtained. It may be relevant that in a book by Romanowsky's contemporary, Professor M. N. Nikiforov, the following statements appear concerning his technique: "Effective staining depends on the composition of methylene blue used and, according to Gautier, the most reliable methods use methylene blue from Badisches Soda-Anilin Fabric, with suffixes C and BGN; together with eosin, with suffix A, from the same manufacturer." Perhaps the presence of mold or the composition of the glass from which the "large bottle" was made also affected oxidation.

The methodological section of Romanowsky's thesis also contains a detailed account of investigations of malaria parasites. The life cycle of the protozoan, and the impact of quinine and extracts of sunflower on these organisms was described. Therefore, Romanowsky not only developed the technique of staining, but also used it to carry out several research studies. The methodological part of the thesis is available on the internet (Romanowsky 1891a).

After Romanowsky had defended his thesis, its main results were published in a German translation in two issues of the journal, *St. Petersburger Medizinische Wochenschrift* (Romanowsky 1891b, c).

This article is well known and has been cited widely outside Russia. A colored chart with drawings of red blood cells containing the malaria parasite at different stages of development was included in both the thesis and publication (Romanowsky 1891b,c). Apparently, these images made a great impression on Romanowsky's contemporaries.

Malachowski's publication

Malachowski's paper concerning polychrome stains was published in August 1891. Because this paper was not available to me, the following remarks are based on the account of Malachowski's work described in an article by R. D. Lillie (Lillie 1978). Before detailing the technical and scientific aspects of Malachowski's work, it is of interest to note that Lillie commented on the question its scientific priority: "Perhaps, we should refer to this family of stains as Malachowski stains? He did introduce deliberate alkali polychroming of the methylene blue eosin blood stains." A number of other articles concerning Malachowski have come from K. Krafts (Krafts 1993, 2002, 2011), sometimes with co-authors (Krafts et al. 2011a,b, Krafts and Pambuccian 2011, Ward et al. 2011). Here, however, I shall be guided by the R. D. Lillie primary publication.

Lillie argued that Malachowski achieved repeatable results by using a stain that involved a freshly prepared aqueous mixture of eosin and methylene blue to which borax had been added. Unfortunately the borax concentration was not given nor was it clear whether the borax solution was prepared by the author or was purchased (Lillie 1978). In any event, after staining with this preparation, the following observations were reported: "blue plasmodia with red-purple chromatin bodies, dark-purple leukocyte nuclei with pale-violet cytoplasm."

To facilitate analysis and discussion, the methods of Romanowsky and Malachowski the outcomes obtained are summarized in Table 1. Below, comments on this information are provided for each of the numbered items in the table.

1. The most important finding was the discovery of a new type of polychrome staining. Scientific priority for this phenomenon, based on the dating of the publications, belongs to D. L. Romanowsky.

2. Despite the excellent staining outcomes, i.e., visualization of the internal structure of malaria parasites including their nuclei with chromatin, which in Romanowsky's case can be seen in the illustrations in his thesis, this was not particularly significant in the scientific sense. Both Romanowsky and Malachowski only confirmed observations of nuclei in malaria parasites, which already had been reported by Italian investigators (Celli e Guarniere 1888/1889). Romanowsky acknowledged this in the final part of his first article; however, the demonstration of the ability to identify the structure of the malaria parasite in the dried preparations with a simple procedure of polychrome staining was extremely important for practical diagnostics of malaria. The priority for this belongs to D. L. Romanowsky.

3. Priority for the use of aged (old) methylene blue solution together with eosin for polychrome staining also belongs to Romanowsky. After his work, many researchers, from Malachowski (1891) to Lillie (1978), used aged methylene blue solutions.

4. Priority of the use of borax and, more widely, the use of alkalization for polychroming methylene blue, belongs to E. Malachowski. Note, however, that prior to Małachowski, Plehn F (1890) had used methylene blue with potassium hydroxide added and Sahli H (1885) had used methylene blue with borax added. Moreover, Malachowski (1891) did not describe how the polychrome methylene blue solution with borax was prepared, as acknowledged by Lillie (1978).

Table 1. Scientific results and technical procedures reported by Romanowsky and Malachowski, 1890–1891

Key observations and technical methods	Romanowsky publication date	Malachowski publication date
1 Polychrome staining of blood preparations containing malarial parasites, using two dyes: eosin and polychromed methylene blue	December 1890	August 1891
2 Staining of malaria parasites with the identification of their internal structure, including nuclei with chromatin	December 1890	August 1891
3 Use of aged methylene blue solution for the preparation of the stain mixture	30 May 1891	Unknown
4 Use of stain with eosin/methylene blue solution with the addition of borax (alkalization of solution)	Unknown	August 1891

Moreover, Donaldson et al. (1978), having studied the kinetics of the oxidation of the methylene solution in aqueous solution containing borax, suggested that: "It seems evident that Malachowski (1891) must have used his routine Sahli stain after it had been on the laboratory shelf for some weeks or months, or after some really hot weather at the time." It is of interest that one of authors of this article was R. D. Lillie. Note also that azures A and B can be obtained also by oxidation of methylene blue in an acidic medium (Lillie 1942, 1969).

Long-term influence of the Romanowsky and Malachowski investigations

D. L. Romanowsky performed and published a number of studies of malaria parasites using his new staining technique. These investigations included a preliminary report, a thesis and several subsequent articles, including articles on marsh diseases in the medical journal, *Vrach* (Romanowsky 1891d, 1892).

A number of well-known investigators subsequently referred directly to Romanowsky's studies, sometimes using the term, "Romanowsky stain," in the titles of their articles: Nocht (1898), Leishman (1901), Reuter (1901), Wright (1902), Giemsa (1904, 1934), May (1906) etc.

Romanowsky's technique, with subsequent modifications, was included in various contemporary books, such as the various editions of M. N. Nikiforov's textbook on microscopic technique (Nikiforov 1919). Another perspective on the impact of Romanowsky's work comes from the founder of the Biological Stain Commission, H. J. Conn, who in 1930 wrote in a review article that:

> If asked who was the originator of such blood stains the average biologist today would undoubtedly cite Romanowsky (1891) whose classic work on staining the malaria organism with a mixture of eosin and methylene blue has served to attach his name to all stains of this type … His outstanding contribution seems rather to have been the demonstration of the nucleus of a parasite in the blood with this compound stain, as the result of which interest in such stains was greatly stimulated. This is undoubtedly the reason why modern blood stains, differing almost as much from his as from Ehrlich's earlier neutral stain combinations, are generally known as modifications of Romanowsky. Moreover, Romanowsky was probably the first to obtain the blood picture which we regard as typical today and which a satisfactory modern blood stain is expected to give regularly.

Unlike Romanowsky, Malachowski wrote only one article on the topic, which developed few links by other scientists (Malachowski 1891). Moreover, "Unfortunately, Malachowski's seminal discovery was ignored, and he never wrote another article on blood staining" (Krafts at al. 2011a). Probably for these reasons there was, according to Lillie (1978), only one reference to Malachowski's article in the period 1891–1978.

Both formal priority for polychrome staining of blood preparations by a solution of eosin plus methylene blue with its oxidation products (azures B and A) and the development of this technique belong to D. L. Romanowsky. His work showed the possibility of using simple methods to obtain polychrome blood preparations with high contrast and resolution that were suitable for identifying the structure of malaria parasites. This was the starting point for the development of polychrome staining techniques and subsequent scientific microscopy examinations for a long sequence of researchers. Ernst Malachowski, however, had priority for obtaining polychrome methylene blue using an alkalized solution of methylene blue.

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