

## Histochemistry for nanomedicine: Novelty in tradition

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### ABSTRACT

During the last two centuries, histochemistry has provided significant advancements in many fields of life sciences. After a period of neglect due to the great development of biomolecular techniques, the histochemical approach has been reappraised and is now widely applied in the field of nanomedicine. In fact, the novel nanoconstructs intended for biomedical purposes must be visualized to test their interaction with tissue and cell components. To this aim, several long-established staining methods have been re-discovered and re-interpreted in an unconventional way for unequivocal identification of nanoparticulates at both light and transmission electron microscopy.

**Key words:** histochemistry; immunohistochemistry; nanoparticles; light microscopy; transmission electron microscopy.

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## Introduction

Since the pioneer book by Francois-Vincent Raspail in 1830,<sup>1</sup> histochemistry has been developing for about two centuries, playing a primary role in biological and medical research. By revealing *in situ* the molecular organization of cell and tissue, histochemistry has indeed provided significant advancements in the knowledge in many fields of life sciences.<sup>2,3</sup>

At the end of the last century, in parallel with the great development of biomolecular techniques, histochemistry has become progressively neglected, being perceived by non-histochemists as a merely descriptive and old-fashioned approach (the term histochemistry being “commonly perceived as an archaic term primarily associated with stains and staining techniques”, in Raimond Coleman’s words).<sup>4</sup> However, fashions change and, during the last decade, histochemical techniques have been reappraised. In fact, the knowledge of the chemical composition and molecular interactions of chemical species in a biological system must be supported by the information on the location and dynamics of specific molecules in cells and tissues. Histochemistry is nowadays more and more oriented toward the detection of single molecules in the very place where their structural and functional roles are exerted.<sup>5</sup>

It is worth noting that histochemical stainings and techniques that were introduced in the first years of the 20<sup>th</sup> century or even before are still routinely used as irreplaceable tools to detect different substances *in situ*.<sup>6</sup> The Perls’ Prussian blue method was introduced in 1867<sup>7</sup> and still is the method of choice for visualizing iron; classical histochemical techniques to demonstrate the presence of lipids<sup>8</sup> or polysaccharides<sup>9</sup> are still popular; Von Kossa’s method<sup>10</sup> to demonstrate the presence of calcium deposits in tissues was developed in 1901 and is presently employed to study the process of mineralization. Remarkably, its long-term impact on life sciences and medicine still places histochemistry at the research forefront in these disciplines.

As a matter of fact, classical histochemical methods have recently been applied in the relatively new and fast-developing field of nanomedicine where the novel nanoconstructs for biomedical purposes must be visualized to test their interaction with various biological systems (from cells in culture, to explanted tissues, to living animals<sup>11-15</sup>).

In the attempt to identify the nanoparticulates inside organs, tissues and cells, researchers re-discovered and re-interpreted in an innovative way several long-established staining methods for unambiguous identification of their molecular constituents at both light microscopy and transmission electron microscopy (TEM).

## Histochemistry for nanomedical research

The most common method to make visible a nanoconstruct at light microscopy consists in loading/linking a fluorescent dye during the synthetic process. Fluorescently labelled nanoparticles may be visualized at both bright-field microscopy and TEM by applying the procedure of diaminobenzidine (DAB) photooxidation. This histochemical method was proposed by Maranto in 1982<sup>16</sup> to convert the fluorochrome signal into a stable reaction product visible at bright-field microscopy as a brownish pigment and at TEM as an electron dense granular precipitate. DAB photooxidation proved to be suitable for different types of nanoparticles conjugated with different fluorophores, allowing their detection in the intracellular milieu even after long time from their uptake.<sup>17-21</sup>

Fluorochrome labelling of nanoconstructs cannot sometimes be used due to technical or experimental reasons: *e.g.*, the rapid loss of the loaded fluorophore in the biological environment may

make the nanoparticles undetectable; or fluorophore addition may alter the original physicochemical properties; or high tissue autofluorescence is incompatible with fluorochrome labelling of the nanoparticles. In all these cases, histochemical staining techniques may provide suitable detecting solutions.

Iron-, gold- or, more generally, metal-based nanoconstructs are easily observed at TEM, due to their intrinsic electron density that makes them unequivocally recognizable in the biological environment (for a recent review see<sup>22</sup>). However, these nanoparticles have no intrinsic feature that may make them visible at light microscopy.

Prussian blue staining has frequently been used to visualize at bright field microscopy iron-based nanoparticles inside cultured cells or tissue slices.<sup>23-28</sup> After treatment with an acidic solution of potassium ferricyanide, iron in the ferric state gives rise to a bright blue pigment called ‘Prussian blue’ (ferric ferrocyanide). The blue pigment can be transformed into a brown-stained product by irradiation with ultraviolet light: this is an especially convenient procedure to improve the detection of low number of iron-based nanoparticles.<sup>29</sup> Gold nanoconstructs may be seen at light microscopy by applying the silver-enhancement technique: the deposition of silver on the gold particles makes them grow in size and become visible under a standard bright-field microscope.<sup>30-33</sup>

Organic nanoconstruct are especially difficult to detect especially at TEM, where they can hardly be discriminated from the cell or tissue environment, due to their low intrinsic electron density.

The critical-electrolyte-concentration Alcian blue method was originally proposed in 1975 by Schofield *et al.* to reveal glycosaminoglycans in tissue sections,<sup>34</sup> and was recently repurposed, in the frame of a nanomedical study, to label with high efficiency and specificity hyaluronic acid-based nanoparticles<sup>35</sup> inside cultured cells at both bright field microscopy (as a blue product) and TEM (as fine electron dense precipitates).<sup>36</sup> Alcian blue has also been used to detect nanoscaled dendritic polyglycerol sulfate amine in liver after *in vivo* injection thanks to the affinity of this dye for the negatively charged sulfate groups.<sup>37</sup>

Various histochemical methods have been proposed to label lipid-based nanoparticles, depending on their chemical nature. Osmium tetroxide is an efficient, long-established fixative for lipid molecules thanks to its addition to the double carbon-carbon bonds of unsaturated fatty acids<sup>38,39</sup> and can also be used as a “dye”: the deposition of metallic osmium in the lipid-containing structures results in an intense brownish or black color at bright-field microscopy, and gives a marked electron density at TEM. Consequently, lipid-based nanoparticles have frequently been visualized by osmium tetroxide.<sup>21,40-44</sup> Lipid nanoparticles have also been made visible at light microscopy by staining with PKH67: this is a fluorescent dye for specific and long-lasting labelling of cell membrane thanks to its long aliphatic tails that ensures stable incorporation into the lipid membrane regions.<sup>43,45</sup> An interesting approach to allow visualization of liposomes in three-dimensional optical microscopy has been set up by Syed *et al.*:<sup>46</sup> to solve the problem of lipid denaturation in cleared tissues, they developed cross-linkable tags that remain attached to the liposome surface in living organs but, when the tissue undergoes fixation, they become cross linked into the tissue, thus revealing the distribution of liposomes.

Rare-earth-based nanoparticles have been visualized at bright-field microscopy in both cultured cells and tissue slices by means of a chromogenic reaction with Chlorophosphonazo III, which gives rise to a blue product independently of the rare earth type, and can be quantitatively evaluated.<sup>47</sup>

Besides “classical” histochemical staining methods, immunohistochemistry too has been used to investigate nanoparticulates

designed for biomedical applications. Immunogold labelling allowed the intracellular localization at TEM of nanocarriers loaded with digoxigenin-containing DNA<sup>48</sup> as well as the unequivocal detection of nanovesicles by targeting membrane markers.<sup>49-51</sup> Chitosan nanoparticles were detected at both fluorescence microscopy and TEM by immunolabelling the loaded drug, tracking also its release in the intracellular compartments.<sup>52</sup>

## Concluding remarks

The above examples are evidence that histochemistry is far from being outdated but still has many responses to give, even in cutting-edge research fields. In nanomedicine, histochemists will find stimulating challenges to test their skill and creativity: established staining techniques will surely find novel applications, and the innovative materials used to manufacture the nanoconstructs will encourage the development of original staining protocols.

Once again, histochemistry will prove to be alive and kicking.

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