Adapted silver impregnation protocol for the detection of Merkel cells in fish skin embedded in plastic historesins

André Luiz Veiga Conrado[1] & José Roberto Machado Cunha da Silva[1]

[1] Laboratory of Evolutive Histophysiology, University of São Paulo, São Paulo, Brazil

Correspondence: André Luiz Veiga Conrado, Laboratory of Evolutive Histophysiology, University of São Paulo, São Paulo, Brazil. E-mail: andreveigaconrado@gmail.com

Received: November 13, 2025 DOI: 10.14295/bjs.v5i1.813

Accepted: December 08, 2025 URL: https://doi.org/10.14295/bjs.v5i1.813

Abstract

Merkel cells are detected using silver impregnation in skin samples embedded in paraplast. Here, we describe an adapted protocol for detecting Merkel cells in Amur carp (*Cyprinus rubrofuscus*) skin samples embedded in historesin. Incubated slides in Grimelius silver impregnation solution at 60 ºC for five hours resulted in positive Merkel cells scattered among skin cell layers composed of Malpighian, goblet, and club cells. Thus, Merkel cells can be explored in samples embedded in historesins using adapted protocols for paraplast ones.

Keywords: argyrophil silver impregnation, fish skin, histologic resin, light microscopy, Merkel cell.

Protocolo adaptado de impregnação por prata para detecção de células de Merkel na pele de peixe emblocada em historresinas plásticas

Resumo

As células de Merkel são detectadas por impregnação de prata em amostras de pele embebidas em historesina plástica. Neste trabalho, descrevemos um protocolo adaptado para detectar células de Merkel em amostras de pele de carpas-de-Amur (*Cyprinus rubrofuscus*) embebidas em historesina. Lâminas incubadas em solução de impregnação de prata de Grimelius a 60 °C por cinco horas resultaram em células de Merkel dispersas entre as camadas de células da pele, compostas por células de Malpighi, caliciformes e claviformes. Portanto, as células de Merkel podem ser investigadas em amostras embebidas em historesina, utilizando protocolos adaptados a amostras em resinas plásticas.

Palavras-chave: impregnação de prata argirofílica, pele de peixe, resina histológica, microscopia óptica, célula de Merkel.

1. Introduction

The diffuse neuroendocrine system is a set of single or clustered scattered endocrine cells that are found along many organs and systems that share biochemical, cytological, and secretory properties, as well as control mechanisms (Montuenga et al., 2003). The skin is settled by neuroendocrine cells located in the epidermal and dermal layers, such as Merkel and mast cells (Slominski, 2005). As part of the cutaneous sensory system, Merkel cells are found in almost all vertebrates and synthesize a wide range of neurotransmitters stored in cytoplasmic granules, which, once released, are responsible for transforming noxious stimuli into action potentials in the afferent nerve fibers (Day; Salzet, 2002; Tachibana; Nawa, 2002; Halata et al., 2003). In fishes, Merkel cells are found in Agnatha, Dipnoi, in some representative orders of Teleostei (Cypriniformes, Siluriformes, Anguilliformes, Scopaeniformes), and in some others, but not in Mixini, in cartilaginous fish, and in bony nonteleostean fish (Kasumyan, 2011).

Regarding morphological characteristics, Merkel cells are not distinguished from other cell types using ordinary stains in light microscopy, such as hematoxylin and eosin (DeLellis, 2001). Therefore, more accurate procedures were described to detect Merkel cells, such as using vital and fluorescent dyes (Nurse; Farraway, 1989; Kotrschal et al., 1993), immunohistochemistry (Tachibana, 1995), and silver impregnation techniques (Grimelius,

2004; Ramírez; de los Monteros, 2019). Allied with stain procedures, the classic embedding medium in histology is paraffin/paraplast, and the challenge is to develop adapted protocols for samples embedded in glycol methacrylate–based resins for light microscopy.

Here, we describe an adapted Grimelius argyrophil silver impregnation method (Grimelius, 2004) to detect Merkel cells in fish skin samples embedded in glycol methacrylate histologic resin.

2. Materials and Methods

2.1 Experimental location

The experiment was conducted at the Laboratory of Evolutionary Histopathology of the University of São Paulo, Brazil, in 2024, at the Institute of Biological Sciences (ICB).

2.2 Experimental sample

Fifty-six juvenile female Amur carps *Cyprinus rubrofuscus* (Lacépède, 1803) (27.20 ± 9.63 g) were kept in a 500 L aquarium at the Aquatic Animals facility of the ICB/USP. Amur carps were fed omnivorous fish chow (360-AM, AMICIL S/A, Brazil) once a day until apparent satiety. During thirty-day acclimation, water physicochemical parameters were daily evaluated: temperature (19.79 ± 1.34 ºC), pH (7.47 ± 0.09), total and toxic ammonia (0.93 ± 1.10 mg L^{-1} and 0.0015 ± 0.0021 mg L^{-1}, respectively), nitrite (1.32 ± 0.75 mg L^{-1}) and dissolved oxygen concentrations (10.52 ± 1.10 mg L^{-1}) using rapid test kits (Alcon Pet®, Brazil).

2.3 Methodological procedure

Amur carps were euthanized in benzocaine-based overdose solution (250 mg L^{-1}) at 20 ºC for 4-5 min, with death confirmed when each carp had a lack of gill movement and no reflex after a noxious stimulus (AVMA, 2013). Skin samples with approximately 1 cm x 1cm x 0.5 cm were collected from the lateral body region and fixed in a 4% paraformaldehyde solution in 0.1 M phosphate buffer.

Samples were fixed for 24-36 h at room temperature and underwent dehydration in graded alcohol solutions until pure resin solution. Skin samples were embedded in historesin (HistoResin, Leica, Germany) in order to separate skin layers. Sections with 3 µm thickness were rinsed in silver nitrate solution for argyrophil stain reaction following Grimelius technique (Grimelius, 2004, protocol #3). The couplin jar was sealed with plastic film (Parafilm® M, Sigma Aldrich, USA) and transferred to a convection oven at 60 ºC for five hours.

Slides were developed in 2% (*w/v*) hydroquinone solution in distilled water at room temperature for five minutes and washed in tap water for 10 min. No counterstain was performed. Slides were dried in a convection oven at 40 ºC and sections were protected with mounting medium (Entellan™, Sigma Aldrich, USA) and glass coverslips.

The histological evaluation was carried out using 40x and 100x objectives of the Axio A1 Scope light microscope (Zeiss, Germany), and the photomicrographs were captured by the AxioCam HRc Camera (Zeiss, Germany).

2.4 Ethics committee

This research was approved by the Ethics Committee on the Use of Animals of the Instituto de Ciências Biomédicas da Universidade de São Paulo (ICB/USP), São Paulo, Brazil # 5420020819.

3. Results

The epidermis of Amur carp (*C. rubrofuscus*) is composed of multilayers of small non-keratinized stratified squamous filament cells (or Malpighian cells) with 6-8 cell thickness, club cells (Figure 1a), and goblet cells (Figure 1b).

With Grimelius argyrophil silver impregnation protocol, it was possible to detect the neurofibrils in the stratum spongiosum of the dermis (SS, Figure 1c) and the delimitation of ovoid cells in the epidermis with silver deposits in the peripheral cytoplasm surrounding the inner plasma membrane (Figure 1d). Due to their morphological characteristics, those cells were defined as Merkel cells (Figures 1e and 1f) surrounded by Malpighian and club

cells.

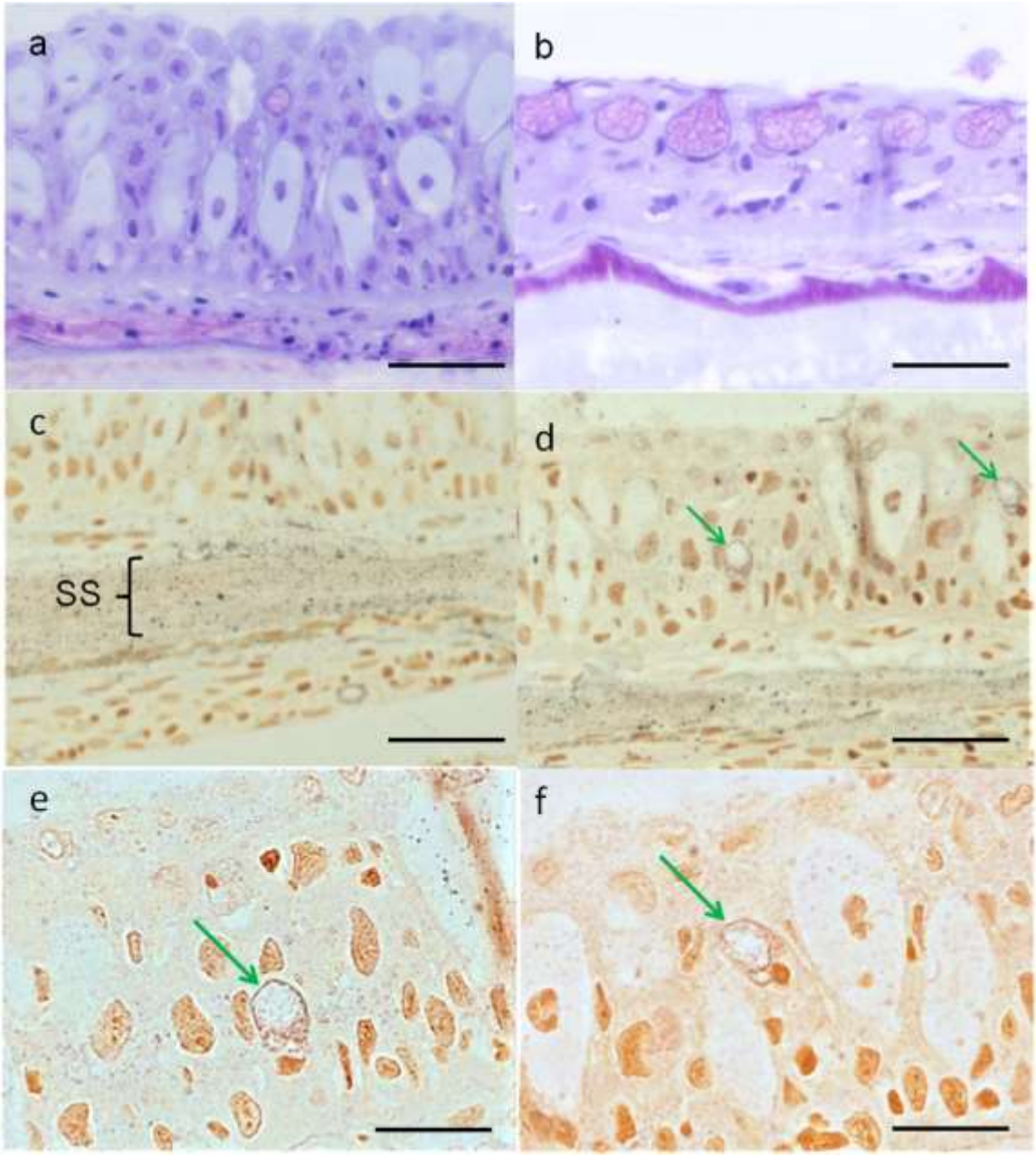

Figure 1. Photomicrographs of the skin of Amur carp (*C. rubrofuscus*). Clustered club cells (**a**) and goblet cells (**b**) were seen among structural Malpighian cells. Neurofibrils are evidenced by silver granules located in the stratum spongiosum (**SS**) of the dermis (**c**). In the epidermis, silver was deposited on the plasma membrane of ovoid cells (arrows) observed between Malpighian and club cells (**d**). Higher magnification of the epidermis revealed that those are Merkel cells (**e** and **f**). In Figure 1e, a Merkel cell (arrow) is surrounded by Malpighian cells. In Figure 1f, observe the proximity between the Merkel (arrow) and club cells. Toluidine blue stain (a and b), Grimelius argyrophil silver impregnation (c – f). Bars: a - d = 50 µm, e and f = 20 µm. Source: Authors, 2024.

4. Discussion

Fish skin is the first barrier against a wide variety of pathogens, predators, and environmental changes. Among the multilayer fish skin epithelial cells (filament or Malpighian cells), goblet cells release acid and neutral

glycoproteins to keep a viscous protection on the outer skin surface (Shephard, 1994), and club cells that store proteins and acidophilic substances, which are released to warn conspecifics of the predator's presence (Rakers et al., 2010).

Belonging to the cutaneous sensory system, Merkel cells are classified as type I slowly adapting receptors and are highly sensitive and react to light touches accompanied by a small deflection or pressure (Kasumyan, 2011). Beyond touching, Merkel cells in common carp *C. carpio* skin react against exposure to acid water (Iger; Wendelaar Bonga, 1994), copper (Iger et al., 1994a), and cadmium solutions (Iger et al., 1994b). Here, we described Merkel cells in Amur carp *C. rubrofuscus*, the wild form of the koi carp *C. carpio* (Bogutskaya et al., 2008; Zhou et al., 2004), with similar gross characteristics and distribution as described in electron microscopy by Tachibana et al. (1984) in *C. carpio* skin samples. Also, the proximity between the club cells and Merkel cells in the *C. rubrofuscus* skin suggests a paracrine regulation of the latter by the former as proposed by Weihe et al. (1998).

Merkel cells can be distinguished from melanocytes and Langerhans cells of the epidermis by electron microscopy (Halata et al., 2003) using morphological criteria reported by Lane & Whitear (1977), with the appearance of granules dependent on the method of fixation (Whitear; Lane, 1981). In light microscopy, positive detection of Merkel cells using an adapted Grimelius argyrophil silver impregnation protocol was described in dog sinus hair follicles (vibrissae) by Ramírez & de los Monteros (2019). Those authors observed strong silver developing with double-impregnation in samples embedded in paraffin.

In our case, thin sections in historesin incubated in the conventional oven for a longer time showed a delicate silver deposition in cytoplasmic granules in Merkel cells due to its presence of serotonine (Zaccone, 1986; Lundqvist et al., 1990). Indeed, a little silver background was achieved with historesin when the developing step was performed only with 2% hydroquinone solution. Moreover, other methods for Merkel cell detection are time-consuming when using vital dye (Nurse; Farraway, 1989) and fluorescent dye (Kotrschal et al., 1993) techniques; however, in immunohistochemistry, expensive technical adjustments may likely be necessary for successful Merkel cell detection in fish samples (Zaccone et al., 1994; Tachibana, 1995). Corroborating with the adapted protocol efficiency, in the pathology laboratory routine for human samples, the Grimelius method is in the workup of neuroendocrine tumors than other stains (DeLellis, 2001; Grimelius, 2008).

Taken together, our results expand our knowledge of the distribution and morphology of Merkel cells in *C. rubrofuscus*, confirming patterns previously observed in *C. carpio* and suggesting a possible paracrine regulation mediated by club cells (Mazzoni; Quagio-Grassiotto, 2020, 2021). Furthermore, we demonstrate that the adapted Grimelius protocol applied to historesin sections is efficient for the detection of these cells, offering a viable, less toxic alternative applicable even to rare or reprocessed samples (Rajan; Malathi, 2014). Thus, this study contributes both to the functional understanding of cyprinid skin and to the advancement of histological methodologies applied to the identification of neuroendocrine cells in fish.

5. Conclusions

In summary, Merkel cells can be detected in light microscopy using a few improvements of the original Grimelius method for samples embedded in historesin, being another useful tool for future studies of those cells' function and distribution.

6. Authors' Contributions

André Luiz Veiga Conrado: conception, data collection, data analysis, drafting the manuscript. *José Roberto Machado Cunha da Silva*: data analysis, interpretation, and final approval of the manuscript.

7. Conflicts of Interest

No conflicts of interest.

8. Ethics Approval

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Funding

This research was funded by the National Council for Scientific and Technological Development (CNPq), Ministry of Science and Technology, Brazilian Government, grants # 159651/2015-8 and 163896/2018-6.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

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