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Technical note

Optimized immunofluorescence method on cleared intact Mongolian gerbil cochlea

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Abstract

Immunofluorescence on cleared intact cochlea allows detailed analysis of the cochlear ultrastructure, while avoiding the problems of dissection and serial sections. Protocols have been developed for mice and Mongolian gerbils. This technical note proposes a detailed and optimized immunofluorescence protocol in the Mongolian gerbil comprising significant quantitative and qualitative improvements. This protocol sequentially comprises: fixation (1 day), decalcification (6 days), pre-treatment (7.5 hours), immunolabelling (42 hours), dehydration and clearing (23 hours), followed by mounting and laser scanning confocal microscopy acquisition. This protocol has been optimized in terms of duration (10 days *versus* 13 days) with a reduction of the number of steps, improvement of the specificity of immunolabelling and optimization of the quality of the results obtained. This technical note provides a detailed description of this protocol.

Key words

Immunofluorescence; Transparency; Clarifying; Cochlea; Hair cells; Mongolian gerbil; Laser scanning confocal microscopy.

1. Introduction

Immunofluorescence on cleared intact cochlea is a long and difficult, but attractive technique in order to analyse the architecture of the intact cochlea without dissection. This technique was described for laser scanning confocal microscopy (LSCM) in 2008 in mice and was adapted to the Mongolian gerbil in 2017 [1,2].

The purpose of this technical note is to present an improved and shorter version of the protocol initially described in 2017 in order to decrease the number of steps and reduce the duration of the procedure, while improving the quality of the results obtained.

2. Technique

2.1. Reagents and equipment

- Phosphate Buffered Saline 10X Solution (10X PBS) (Ref: BP3994; Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.).
- Paraformaldehyde = PFA (Ref: P6148; Sigma-Aldrich),
- Ethylenediaminetetraacetic acid (EDTA) (Ref: E9884; Sigma-Aldrich),
- Triton X-100 (Ref: X100; Sigma-Aldrich).
- Gelatin from cold water fish skin (Ref: G7041; Sigma-Aldrich).
- DAPI – 4′,6-Diamidino-2-phenylindole dihydrochloride (Ref: D8417; Sigma-Aldrich).
- Phalloidin-TRITC – Phalloidin-Tetramethylrhodamine Isothiocyanate (Ref: P1951; Sigma-Aldrich).
- Polyclonal IgG Anti-Neurofilament 200 antibody produced in rabbit (Ref: N4142; Sigma-Aldrich).
- Polyclonal IgG Anti-MYO7A antibody produced in rabbit (Ref: HPA028918; Sigma-Aldrich).

- Polyclonal IgG Prestin (C-16) antibody produced in goat (Ref: sc-22694; Santa Cruz Biotechnology, Dallas, Texas, U.S.A.).
- Purified mouse IgG_1 anti-CtBP2 antibody (Ref: 612044; BD Biosciences, Franklin Lakes, New Jersey, U.S.A.).
- Monoclonal Anti-Parvalbumin antibody (IgG1) produced in mouse (Ref: P3088; Sigma-Aldrich)
- Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate (Ref: A21206; Thermo Fisher Scientific).
- Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 568 conjugate (Ref: A11057; Thermo Fisher Scientific).
- Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 647 conjugate (Ref: A31571; Thermo Fisher Scientific).
- Absolute ethanol (Ref: E7811; Alcogroup, Anet, France),
- Methyl salicylate (Ref: M6752; Sigma-Aldrich),
- Benzyl benzoate (Ref: B6630; Sigma-Aldrich),
- Buffer solution: PBS 1X.
- Fixation solution: 4% PFA in PBS 1X.
- Decalcification solution: 10% EDTA in PBS 1X.
- Permeabilization solution: Triton X-100 0.5% in PBS 1X.
- Blocking solution: PBSGT: PBS 1X with 0.2% gelatin and 0.5% Triton X-100.
- Dehydration solutions: 70% ethanol, 90% ethanol and absolute ethanol (100%).
- Clarifying solution (Spalteholz) = MSBB (methyl salicylate benzyl benzoate): 5 parts of methyl salicylate for 3 parts of benzyl benzoate.
- 10 mL transparent glass vials (Ref: 11717617; Thermo Fisher Scientific)
- 10 mm polyethylene clip caps (Ref: 11797606; Thermo Fisher Scientific)

- 0.2 mL Eppendorf tube (Ref: 72.737.002; Sarstedt AG& Co., Nümbrecht, Germany)
- µ-slide 8 Well ibiTreat (Ref: 80826; ibidi, Munich, Germany).

2.2. Detailed protocol

Each freshly harvested cochlea is processed according to an approximately 10-day protocol, comprising the steps indicated in **Table 1**. Primary antibody dilutions are 1:200 (mouse anti-CtBP2) or 1:500 (rabbit anti-NF200 and mouse anti-parvalbumin). Secondary antibodies are used at a dilution of 1:500. Phalloidin-TRITC is used at a concentration of 15 µg/mL and DAPI is used at a concentration of 0.5 µg/mL. These dilutions are purely indicative and must be adapted to each brand of antibody used and to each experimental condition (sample size, exposure time, type of microscope used), possibly after a titration phase. All steps following the addition of fluorophores must be performed in darkness.

Laser scanning confocal microscopy acquisition is performed with an LSM 710 inverted confocal microscope with an EC Plan-Neofluar 10x/0.3 M27 (Carl Zeiss, Jena, Germany) or a Plan-Apochroma 20s/0.8 dry objective or a Plan-neofluar 40x/1.3 or a Plan-Apochroma 63x/1.4 oil immersion objective.

The laser sources used to excite the fluorophores are: 405 nm UV diode laser (DAPI), 488 nm Argon laser (Alexa fluor 488), 561 nm Diode-Pumped Solid-State (DPSS) laser (TRITC or Alexa fluor 568), 633 nm Helium-Neon laser (Alexa fluor 647)

The fluorescence emission signal is detected by photomultipliers: 2 conventional and 1 spectral with a gain of 800 Volts. Acquisitions are performed in Z-stacks (recovery of all optical sections at a defined height) using Zen acquisition software (Carl Zeiss).

2.3. Results

The protocol results in a macroscopically transparent cochlea (**Fig. 1**). The organ of Corti can be analysed on high-magnification views (**Fig. 2**). Dissection-induced trauma may be observed, especially basilar membrane lesions during fixation by cochlear perfusion (**Fig. 3**). These lesions can be avoided by very careful and gentle dissection and perfusion, but can be easily identified whenever they occur.

Finally, the position of the cochlea impacts image quality according to the turn of the cochlea studied, as the quality of image acquisition decreases with increasing distance between the turn of the cochlea and the objective, mainly due to the presence of a greater volume of tissue, which, although transparent, nevertheless induces light diffraction (example in **Fig. 4**).

3. Discussion

The procedure described here constitutes significant optimization of the cleared intact cochlea immunofluorescence protocol in the Mongolian gerbil, comprising both quantitative and qualitative improvements.

Quantitatively, the duration of the protocol and the number of steps have been significantly reduced. The duration of the protocol has been reduced to 10 days versus more than 13 days [2] (6 days of decalcification instead of 7 days; 1 day of primary antibody instead of 3 days; 17 hours of tissue clarifying instead of 22 hours). The number of steps has also been reduced: the Image IT-FX signal amplification step has been eliminated, Phalloidin-TRITC and DAPI are used simultaneously with the secondary antibody solution rather than successively (eliminating four steps, including the intermediate rinsing steps) and elimination of one MSBB bath. The decreased number of steps simplifies the protocol and decreases the risk of errors.

The protocol has also been qualitatively improved in order to obtain better results. As indicated above, the Image IT-FX signal amplification step has been eliminated, as it did not provide any significant qualitative improvement of the images. The increased concentration (0.5% versus 0.1%) and exposure time (2 hours versus 30 minutes) of membrane permeabilization (Triton-X100) facilitates the penetration of specific markers. The blocking agent concentration has been reduced to decrease competition between blocking proteins and antibodies and to improve specific labelling, while continuing to block nonspecific sites. The use of 0.2% fish skin gelatin rather than 10% bovine serum albumin as blocking agent gives better results in terms of blocking of nonspecific binding sites [3]. In parallel, blocking of nonspecific binding sites by immersion in the blocking solution has also been prolonged (4 hours versus 2 hours) in order to improve the specificity of immunolabelling. PBS 1X rinsing times have been systematically increased (three 30-minute immersions instead of three 15-minute immersions), especially after exposure to antibodies (three 1-hour immersions instead of three 15-minute immersions) in order to decrease background noise and aggregates.

The choice of fluorophores is critical, as a maximum of 4 fluorophores can be used in LSCM (quadruple labelling) for several reasons. Firstly, an increased number of fluorophores increases the acquisition time and therefore the risk of photobleaching during acquisition. Secondly, simultaneous acquisition of several fluorophores is associated with a risk of crosstalk (cross-emission and cross-excitation of fluorophores) and fluorescence resonance energy transfer (FRET), when the fluorophore emission spectrum enters the excitation spectrum of another adjacent fluorophore, the emission energy of the first fluorophore is transferred by resonance to the second fluorophore and acts as an excitation energy, thereby decreasing the intensity of the first emission and increasing the intensity of the second emission). These two phenomena can be responsible for errors of interpretation.

Fluorophores with the smallest possible overlap of the excitation and emission wavelengths must therefore be chosen. The fluorophores used in our protocol are: DAPI, Alexa fluor 488, TRITC, Alexa fluor 568 and Alexa fluor 647 (Table 2). They can be used simultaneously except for Phalloidin-TRITC and Alexa fluor 568, which present overlapping spectra.

Alexa fluor fluorophores are preferred to Atto fluorophores because they present high fluorescence emission, limited photobleaching and narrow excitation and emission spectra, making them very useful for double or triple labelling [4, 5].

Some of the elements targeted in this protocol were already targeted in the original protocol [2]: nuclear DNA by DAPI, cytoskeleton actin by Phalloidin-TRITC (phalloidin is a cytoskeleton actin intercalating agent present in all cells, especially stereocilia of hair cells [6]), and neurons by anti-neurofilament 200 kDa antibody.

Other cellular subunits have been targeted in order to improve the specificity of immunofluorescence:

- Parvalbumin: calcium-binding protein mainly present in hair cells and neurons [7].
- CtBP2 protein (Carboxy-terminal binding protein 2): transcriptional repressor of inner hair cell presynaptic ribbons [8], which is only useful at high magnifications.

Other cellular subunits can also be targeted, such as myosin VIIA (present in hair cells [9]) and prestin (present in outer hair cells, it is the motor protein responsible for electromotility of the cochlear amplifier [10]).

Whole-mount cochlea volume acquisition is technically difficult for several reasons: (1) the lowest power objective available (10x) cannot visualize all of the cochlea at the basal turn (requiring the use of mosaics), (2) acquisition over the entire length of the sample (1.5 to 2 mm) takes more than 7 hours, (3) photobleaching of fluorophores can occur due to the long acquisition time and the number of steps required, (4) zones situated furthest from the objective emit much less fluorescence due to photobleaching, but also due to the fact that the

laser beam must cross the entire thickness of the sample, resulting in light diffraction (as the sample is obviously not totally transparent).

The present protocol has therefore been qualitatively and quantitatively optimized, but remains a long and complex procedure. However, the major advantage of this technique is to allow perfectly satisfactory examination of the intact cochlea without dissection.

4. Conclusion

The immunofluorescence protocol on cleared intact Mongolian gerbil cochlea described here has been optimized and simplified to make it accessible to a greater number of teams.

Conflict of interest

None

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Table 1: *Intact cochlea immunofluorescence and clarifying protocol*

Step	***Description***	***Duration***
Fixation		**1 day**
Microdissection	Removal of excess bone tissue, stapedial artery and stapes. Opening of the round window and creation of a small burr hole in the cochlear apex (helicotrema).	
Fixation	Gentle perfusion with 4% PFA in PBS 1X.	
Microdissection	Removal of any remaining excess tissue, enlargement of the apex orifice, and creation of another burr hole in the basal turn to facilitate circulation of reagents.	
Post-fixation	Immersion in 4% PFA in PBS 1X at 4°C for 22 hours.	
Decalcification		**6 days**
Rinsing	Three 30-minute immersions in PBS 1X.	
Decalcification	Immersion in 10% EDTA in PBS 1X at 4°C for 6 days.	
Rinsing	Three 30-minute immersions in PBS 1X.	
Storage	Storage in 70% ethanol at 4°C until subsequent use (not systematic).	
Pretreatment		**7 hours 30 minutes**
Rinsing	Three 30-minute immersions in PBS 1X.	
Permeabilization	Immersion for 2 hours in PBS 1X + 0.5% Triton X-100.	
Blocking	Incubation for 4 hours in blocking solution (PBSGT): PBS 1X + 0.2% Gelatin + 0.5% Triton X-100.	
Immunolabelling		**42 hours**
Primary antibody	Incubation with primary antibody in blocking solution (PBSGT) for 24 hours at 4°C.	
Rinsing	Three 1-hour immersions in PBS 1X.	
Secondary antibodies	Incubation with secondary antibody in blocking solution (PBSGT) + DAPI Phalloidin-TRITC for 12 hours at 4°C, in darkness.	
Rinsing	Three 1-hour immersions in PBS 1X, in darkness.	
Dehydration and clarifying		**23 hours**
Dehydration	Successive baths in graded ethanol: two hours in 70% ethanol, 90% ethanol and absolute ethanol, in darkness.	
Clarifying	Immersion in MSBB mixed with absolute ethanol (1:1) for 4 hours, then 2 successive MSBB baths for 1 hour then 12 h ours, in darkness.	
Mounting		**5 minutes**
Mounting	On iBidi slides with immersion in MSBB.	

PFA = Paraformaldehyde; PBS = Phosphate Buffered Saline; EDTA = EthyleneDiamine Tetraacetic Acid; PBSGT = PBS 1X + 0.2% Gelatin + 0.5% Triton X-100; DAPI = 4',6-Diamidino-2-Phenylindole Dihydrochloride; Phalloidin-TRITC = Phalloidin-Tetramethylrhodamine Isothiocyanate; MSBB: Methyl-Salicylate (5 parts) and Benzyl benzoate (3 parts). All steps are performed in 10 mL glass vials with gentle shaking (30 r.p.m.) at room temperature, except when otherwise indicated. The volume of product used is 5 mL (except for antibody solutions: 2 mL).

Table 2: *Excitation and emission wavelengths of the various fluorophores used.*

Fluorophore	Excitation wavelength (λ_{ex})	Emission wavelength (λ_{em})	Colour of emission spectrum
DAPI-DNA complex	364 nm	454 nm	Violet-Blue
Alexa fluor 488	495 nm	519 nm	Green
Phalloidin-TRITC	540-545 nm	570-573 nm	Yellow
Alexa fluor 568	578 nm	603 nm	Orange-Red
Alexa fluor 647	650 nm	668 nm	Far-red-Infrared

DAPI = 4',6-Diamidino-2-phenylindole dihydrochloride; DNA = Deoxyribonucleic acid; TRITC = Tetramethylrhodamine B isothiocyanate. Note that the visible light spectrum ranges from 400 to 700 nm (ultraviolet below, infrareds beyond).

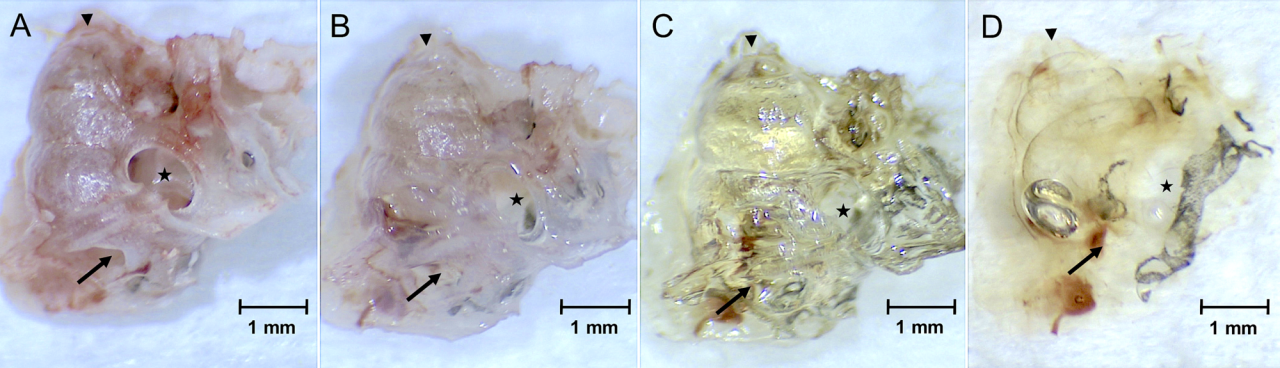
Figure 1: The same left cochlea at the various steps of the tissue clarifying protocol.

A) Immediately after harvesting. B) After decalcification. C) After clearing in air (glass-like appearance). D) After clearing, immersed in MSBB = Methyl Salicylate and Benzyl Benzoate (transparent appearance with good visualization of the three turns of the cochlear spiral). The cochlear apex (arrowhead), the round window (arrow) and the oval window (star) are indicated. Stereomicroscope, 10X objective and x1 magnification. Scale = 1 mm.

Figure 2: Immunofluorescence of the organ of Corti at low (1) and high magnifications (2): *A) DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) nuclear labelling (blue), B) Phalloidin labelling of actin (green). B1 clearly visualizes the V-shaped ciliary tuft of inner hair cells (circle) and outer hair cells (square). Note that the axis of the inner hair cells is perpendicular to that of outer hair cells. C) Neurofilament labelling (red). D) Labelling of Carboxy-terminal Binding Protein 2 (CtBP2) of the presynaptic ribbon (cyan), which can only be visualized at high magnification (D2). E) Image fusion with dotted representation of the shape of an inner hair cell. Laser scanning confocal microscopy. 40X objective. Maximum Intensity Projection. Scale = 10 µm (1) and 5 µm (2).*

Figure 3: Immunofluorescence of an apical turn of the cochlea. A) *DAPI nuclear labelling (blue). B) Phalloidin labelling of actin (green). C) Labelling of hair cells by Parvalbumin (red). Note that the axis of the inner hair cells is perpendicular to that of outer hair cells. D) Image fusion with volume rendering. The zone delineated by dots corresponds to trauma of the basilar membrane during microdissection of the cochlea (creation of a burr hole at the cochlear apex and perfusion). Arrows indicate several zones that lack outer hair cells. DAPI = 4',6-Diamidino-2-phenylindole dihydrochloride. Laser scanning confocal microscopy. 10X objective. Maximum Intensity Projection. Scale = 100 µm.*

Figure 4: The same cochlea, showing the basal (A), median (B) and apical (C and D) turns in different positions *(cochlea placed on its base for A, B and C; cochlea placed on its apex for D) with 1) DAPI in blue, 2) Phalloidin in green, 3) Neurofilament in red, 4) Parvalbumin in cyan, 5) Image fusion. The apical turn visualized from the base (C) has a duller appearance than the apical turn visualized from the apex (D). Similarly, the middle turn (B) has a duller appearance than the basal turn (A) when the laser beam is directed through the base. These findings can be explained by the thickness of tissue crossed by the laser beam. DAPI = 4',6-Diamidino-2-phenylindole dihydrochloride. Laser scanning confocal microscopy. 10X objective. Maximum Intensity Projection. Scale = 100 µm.*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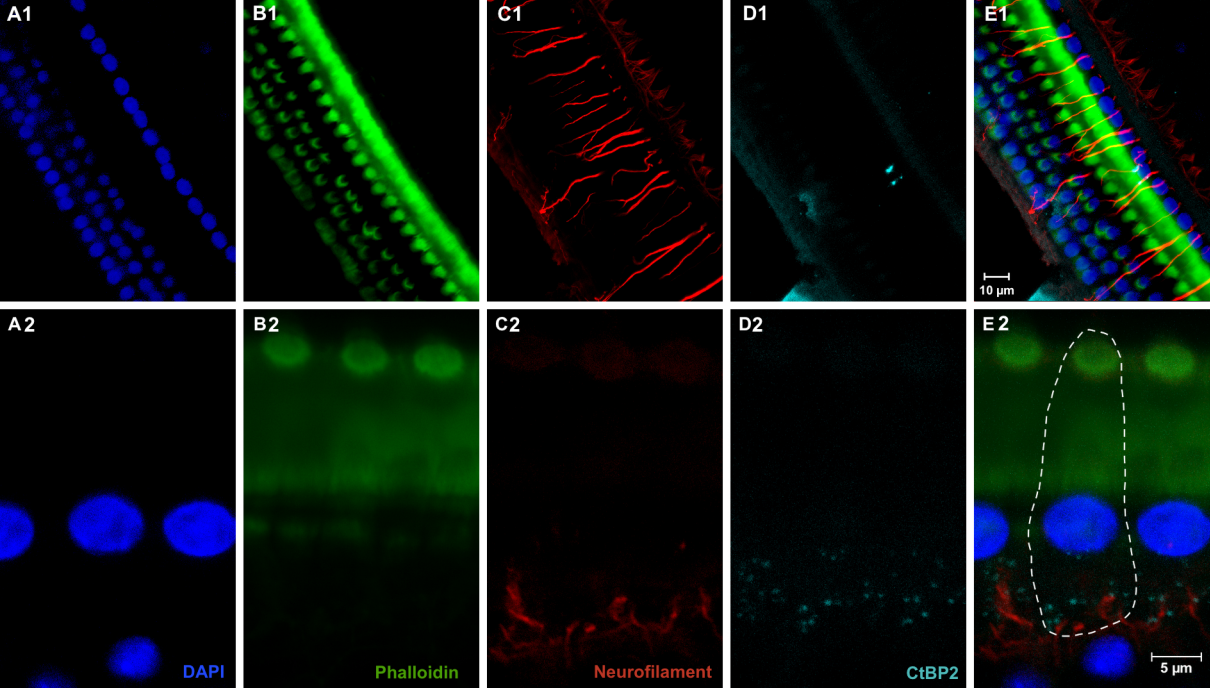
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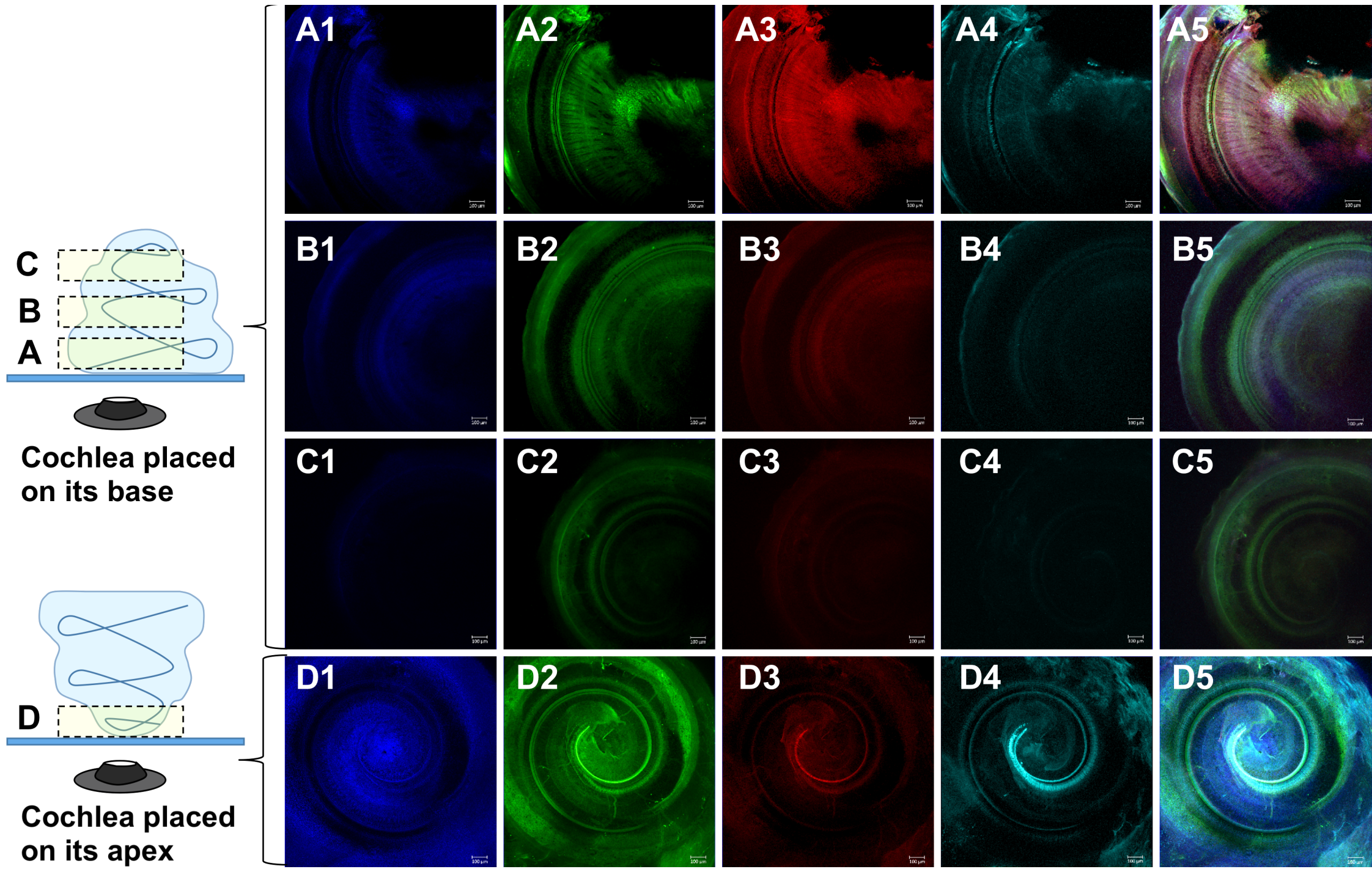
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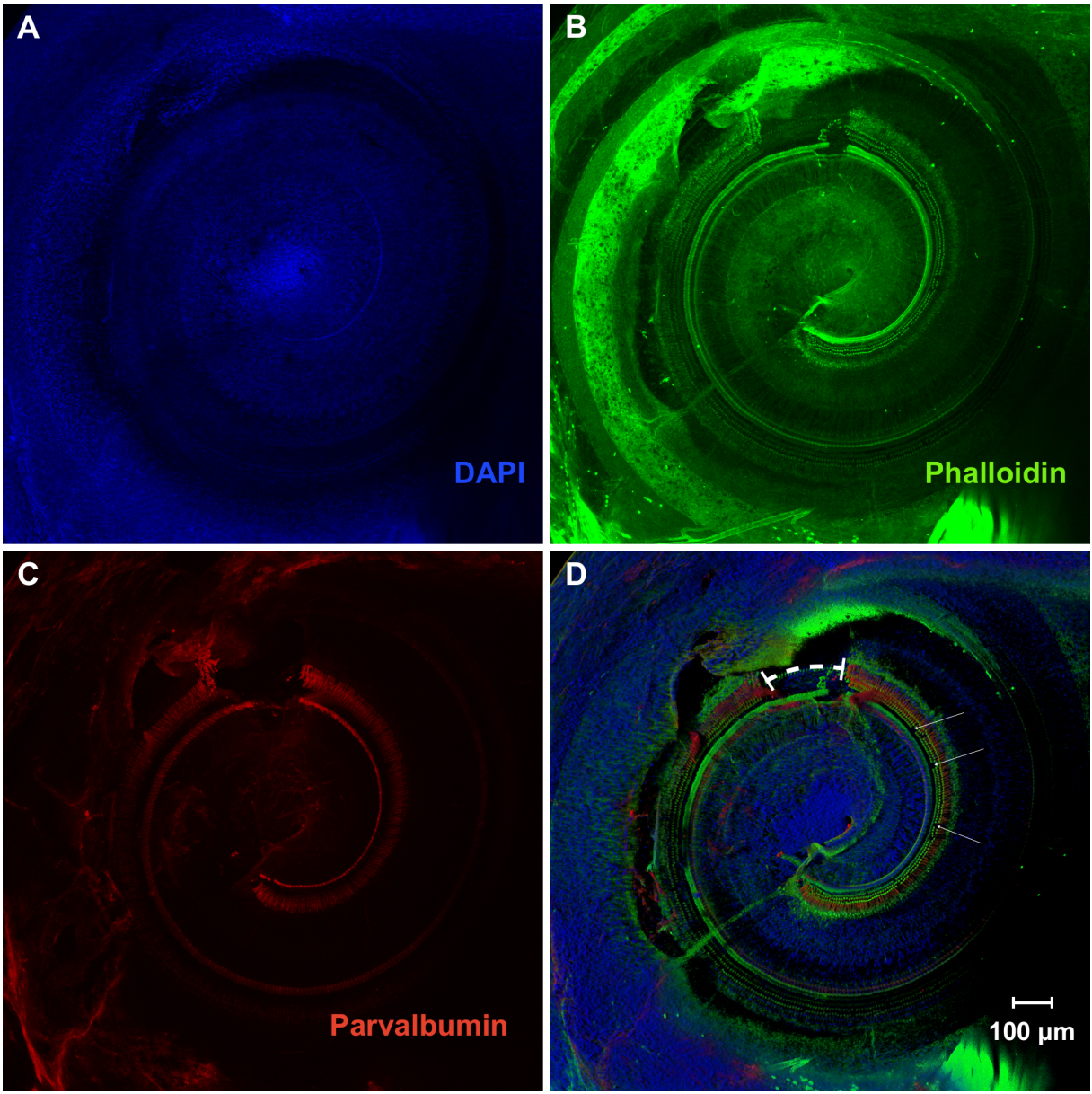
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