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# In Situ Identification of Ectoenzymes Involved in the Hydrolysis of Extracellular Nucleotides

*Mireia Martín-Satué, Aitor Rodríguez-Martínez and Carla Trapero*

## Abstract

Adenosine triphosphate (ATP) and other nucleotides and nucleosides, such as adenosine, are signaling molecules involved in many physiological and pathophysiological processes. The group of cell and tissue responses mediated by these molecules is known as purinergic signaling. Ecto-nucleotidases are ectoenzymes expressed at the cell membrane that act sequentially to efficiently hydrolyze extracellular ATP into adenosine, and they are key elements of this signaling. There is growing interest in studying these enzymes in relation to various pathologies, especially those with an inflammatory component such as cancer. This review summarizes the main protocols for the study of the expression and in situ activity of ectoenzymes in tissue slices and cultured cells.

**Keywords:** nucleotidase, in situ histochemistry, CD39, CD73, lead staining

## 1. Introduction

In this chapter, we intend to detail basic protocols for the in situ detection of ecto-nucleotidases as an introduction to the technique for those who have never made these experimental approaches. This chapter does not aim to be a review on ecto-nucleotidases because there are already excellent highly recommended reviews [1–4].

Ecto-nucleotidases are broadly expressed enzymes active in almost all tissues of all organisms, both animals and plants. What varies among the cell (and tissue) types are the subtype(s) of enzyme(s) and the combination of them, expressed in a particular cell type. In general, these enzymes convert adenosine triphosphate (ATP), as well as diphosphate (ADP) and monophosphate (AMP), into adenosine. In situ detection of these enzymes confers functional sense on immunodetection studies. It is also a convenient tool for the validation of new inhibitors of these enzymes, which can be studied in the cell context of the tissue where they are found. The study of ecto-nucleotidases and their inhibitors (many of them antibodies) is at the center of oncological research to therapeutically target the adenosinergic pathway, a fact reflected in the increased number of high impact publications in the field.

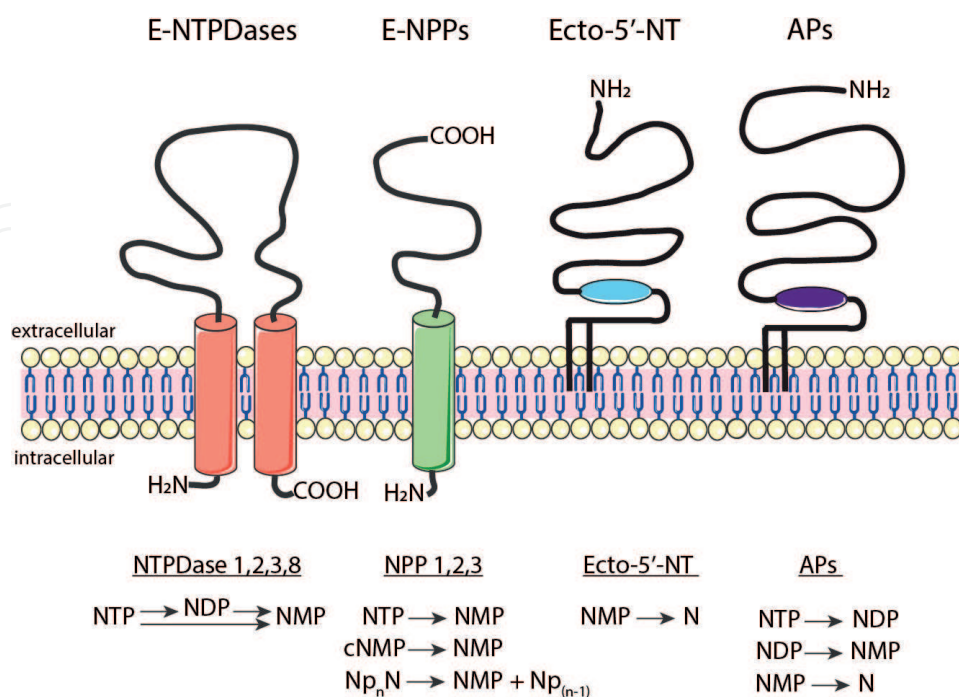
The technique is feasible because ecto-nucleotidases maintain their activity of hydrolyzing nucleotides in formalin-fixed frozen tissues (and cells). Inorganic phosphorous (Pi) generated upon their activity combines with a lead salt added to the reaction mixture, forming brown precipitates in the places where the enzymes are active, which can be visualized under light microscope. The protocol, with slight modifications, can also be used for electron microscopy.

There are four families of membrane-bound ecto-nucleotidases. Other nucleotidases act intracellularly but are not studied here. The main features of ecto-nucleotidases are included in **Figure 1** and summarized below.

### 1.1 Ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases)

The E-NTPDase family is composed of eight members, four of which are cell surface-located: NTPDase1, also known as CD39; NTPDase2 or CD39L1; NTPDase3 or CD39L3; and NTPDase8. They perform the ATP (and ADP) hydrolysis to AMP with different ADP production abilities. These differences between enzymes reflect different consequences in cells depending on the ATP receptors expressed [5]. The four members display similar structural properties, with two transmembrane domains, close to the N and C terminus, and a catalytic extracellular domain [3]. They require millimolar concentrations of  $Mg^{2+}$  and  $Ca^{2+}$  ions in order to perform ATP hydrolysis, and the absence of these ions results in no enzymatic activity. All of them hydrolyze nucleoside triphosphates (NTP), but they differ in substrate specificity. NTPDase1 hydrolyzes ATP and ADP equally, while NTPDase3 and NTPDase8 hydrolyze ATP or uridine triphosphate (UTP) more efficiently than ADP or uridine diphosphate (UDP). Finally, the NTPDase2 is the most ATP-specific NTPDase, and for this reason it is also named the ecto-ATPase [2].

Most of the available NTPDase inhibitors are ATP analogues such as ARL-67156 and PSB-6426, a potent NTPDase2 inhibitor. Non-nucleotide-based inhibitors also described in literature are compounds related to dyes bearing sulfonate groups such



**Figure 1.** Schematic representation of the four families of membrane-bound ecto-nucleotidases and their substrate specificities. E-NTPDases, ecto-nucleoside triphosphate diphosphohydrolases; E-NPPs, ecto-nucleotide pyrophosphatase/phosphodiesterases; ecto-5'-NT, ecto-5'-nucleotidase; APs, alkaline phosphatases; NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; NMP, nucleoside monophosphate; cNMP, cyclic nucleoside monophosphate; N, nucleoside.

as suramin, a nonselective inhibitor, and the pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS). Other inhibitors are the polyoxometalates, such as POM 1 [6]. Inhibitory antibodies, mainly against CD39, are being developed for use in cancer therapy [7].

## 1.2 Ecto-nucleotide pyrophosphatases/phosphodiesterases (E-NPPs)

The E-NPP family represents a versatile group of seven structurally related enzymes with pyrophosphatase and phosphodiesterase activities having a wide range of hydrolysable substrates. The membrane-bound ectoenzymes NPP1 and NPP3 and the secreted NPP2 are the most studied members. Catalytic activity of E-NPPs is composed of a two-step hydrolysis consisting of a first attack on the phosphate of the incoming substrate by a threonine/serine metal-activated catalytic site and a second attack on the intermediate substrate by a metal-activated site, thus releasing a nucleoside 5'-monophosphate. In general, NPP1–3 are typed as alkaline ecto-nucleotide pyrophosphatases that hydrolyze a number of phosphodiester bonds (e.g., from oligonucleotides, lysophosphatidylcholine, sphingomyelin, and glycerophosphorylcholine or from artificial substrates like the p-nitrophenyl 5'-thymidine monophosphate (p-Nph-5'-TMP)) and pyrophosphate bonds (e.g., from (d)NTPs, (d)NDPs, NAD, FAD, and UDP sugars or from artificial substrates like the thiamine pyrophosphate (TPP)) to generate nucleoside 5'-monophosphates. TPP is the “false” substrate mainly used for NPP identification in *in situ* activity assays. Like most of the enzymes, E-NPPs can be inhibited *in vitro* by the substrates and products of the NPP reaction, as well as by heparin and heparan sulfate glycosaminoglycans, and by other substances such as imidazole, 2-mercaptoethanol, and metal ion-chelating agents [8]. Anti-NPP3 inhibitory antibody represents a promising therapeutic tool for the treatment of renal cell carcinoma [9].

## 1.3 Ecto-5'-nucleotidase (ecto-5'-NT, eN)/CD73

Extracellular AMP resulting from the hydrolysis of ATP and ADP by most of the ecto-nucleotidases can in turn be efficiently hydrolyzed into adenosine by eN, a glycosylphosphatidylinositol-linked membrane-bound glycoprotein also known as CD73 [10], which hydrolyses nucleotide-5'-monophosphates (NMP) [3]. It is broadly expressed as an alpha dimer bound with disulfide bridges and shows different functions depending on the cell type. Although eN activity is ion-independent in physiological conditions, *in vitro* the presence of  $Mg^{2+}$  ions can considerably increase its ability to hydrolyze AMP. In addition to its AMPase activity, eN hydrolyzes 2-deoxyribose compounds but much less effectively than AMP. Unlike other ectoenzymes such as NPPs, eN is not inhibited by Pi. Alpha,beta-methylene-ADP and some of its derivatives and analogues are efficient inhibitors [11]. Inhibitory anti-CD73 antibodies are used in clinical trials [7].

## 1.4 Alkaline phosphatases (APs)

Phosphatases are a superfamily of proteins that mediate the phosphate removal of proteins and other substrates [12]. Depending on their substrate specificity, they are divided into two major groups: the protein phosphatases, which mediate the hydrolysis of phosphate groups from protein residues (e.g., serine/threonine phosphatases), and the membrane-bound phosphatases, which mediate the hydrolysis of phosphate groups from nonprotein substrates (e.g., acid and alkaline phosphatases). In this chapter, we are focusing on the membrane-bound phosphatases, in particular the AP family [12, 13].

APs are zinc-containing dimeric membrane-bound glycoproteins that require magnesium ion for the hydrolysis of a wide range of phosphomonoesters. Although optimum activity occurs at alkaline pH (9.3–10.3), they are also active at a physiological pH, and they are primarily responsible for the PPi phosphohydrolysis in neutral and alkaline environments. APs are classified by their tissue expression and distribution [14]; in humans there are four types of APs in two main groups: the tissue nonspecific alkaline phosphatase (TNAP), with only one member, and the tissue-specific APs, which include the placental-like alkaline phosphatase (PLAP), the germ cell alkaline phosphatase (GCAP), and the intestinal alkaline phosphatase (IAP). Despite the fact that TNAP expression is not tissue-specific, it is mainly found in the liver, bone, and kidneys [15].

APs catalyze the hydrolysis of monoesters of phosphoric acids and have extensive substrate specificity *in vitro*. For example, TNAP is able to hydrolyze ATP, ADP, AMP, PPi, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, and  $\beta$ -glycerophosphate; however, only a few compounds have been considered as natural *in vivo* AP substrates, like PPi, pyridoxal-5'-phosphate (PLP or vitamin B5), and phosphoethanolamine (PEA). APs by themselves are extremely efficient ATPase enzymes, but an autoregulatory mechanism has been described in order to modify the substrate specificity depending on the environmental concentrations of free inorganic phosphates or cell and tissue demands. Pi itself is known to inhibit the hydrolytic activity through a competitive mechanism, and therefore Pi levels will impact the ability of AP to hydrolyze PPi [15, 16]. Levamisole is an inhibitor of the APs. Because of the ability to cleave all forms of adenosine phosphates, APs significantly influence purinergic signaling [17].

## **2. In situ nucleotidase activity experiments**

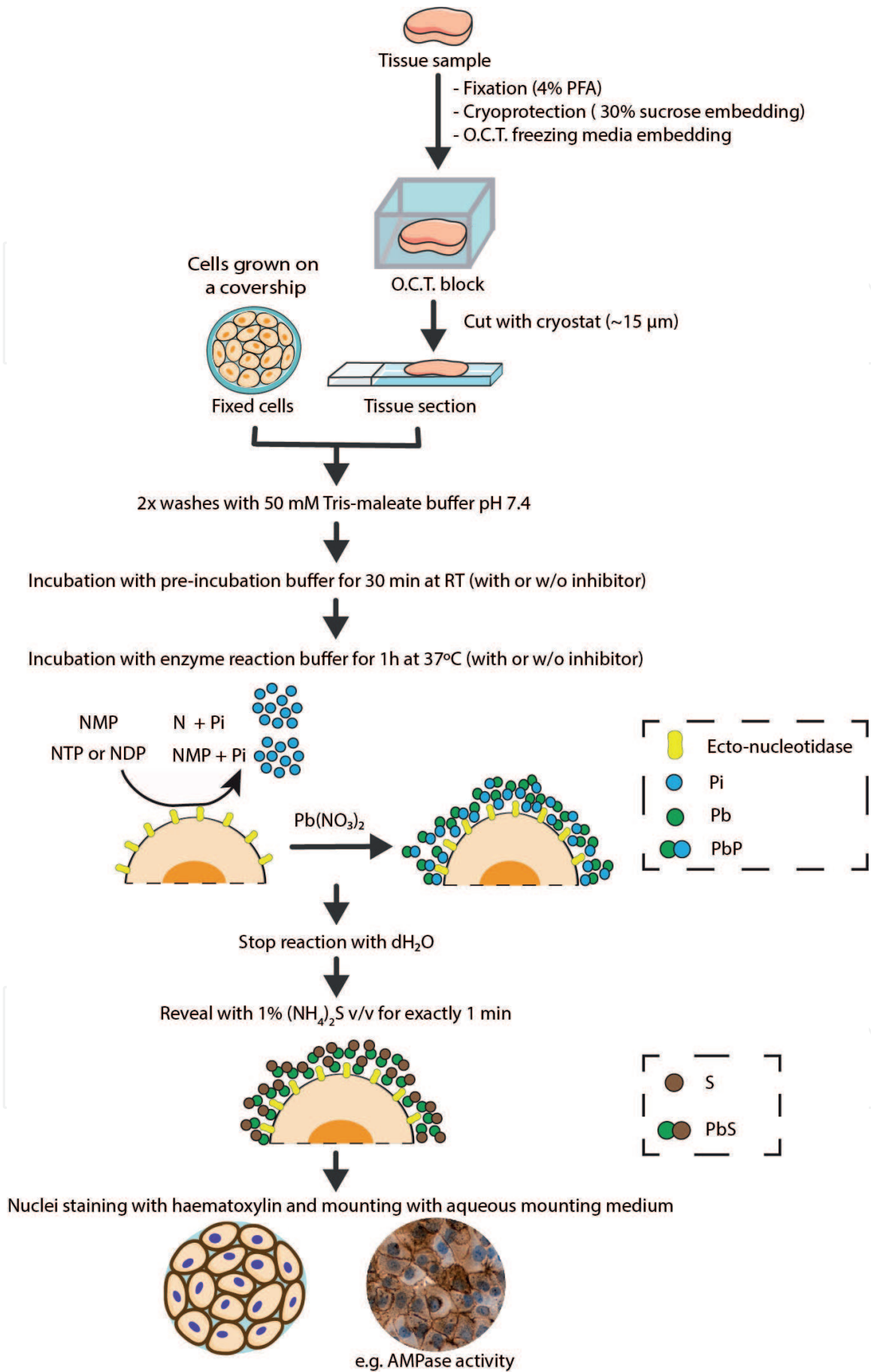
The protocol detailed here for the detection of E-NTPDases, E-NPPs, and eN is based on the Wachstein-Meisel lead phosphate method [18], and the protocol for AP identification is based on the Gossrau method [19], with some modifications.

### **2.1 Wachstein-Meisel lead phosphate-based method**

#### *2.1.1 In tissue samples*

Tissue pieces are fixed with 4% paraformaldehyde for a time period varying from a few hours to 2 days depending on the size (**Figure 2**). Following fixation, tissue pieces are cryopreserved by embedding them in 30% sucrose (in Milli-Q H<sub>2</sub>O) O/N or until tissue sinks. Tissues are then embedded in optimum cutting temperature (OCT) compound and cut with a cryostat into 15  $\mu$ m-thick sections that are put on slides. It is recommended that pretreated slides be used, either homemade polylysine-treated or the commercial ones, to eliminate tissue loss during the procedure. Sections are stored at  $-20^{\circ}\text{C}$  until use.

Tissue slides are rinsed with phosphate buffered saline (PBS) to remove OCT compound and washed twice with 50 mM Tris-maleate buffer pH 7.4 at RT. Slides are then incubated for 30 min at RT with preincubation buffer (50 mM Tris-maleate buffer pH 7.4 containing 2 mM MgCl<sub>2</sub> and 250 mM sucrose) and then for 1 h at 37°C with the enzyme reaction buffer (50 mM Tris-maleate buffer pH 7.4 supplemented with 250 mM sucrose, 2 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 2 mM Pb(NO<sub>3</sub>)<sub>2</sub>, and 2 mM



**Figure 2.** Scheme including the main steps of the lead phosphate-based method for ecto-nucleotidase activity detection.

CaCl<sub>2</sub> and stabilized with 3% Dextran T-250) in the presence or absence of nucleotide as substrate (e.g., ATP). The incubation time and the substrate concentration may vary depending on the experiment, but 1 h at 1 mM is generally suitable. To avoid interference with AP activity, experiments are performed in the presence of the inhibitor levamisole (2.5 mM). Note that the optimum pH for APs is 9, but they are also active at a pH of 7.4. The reaction is stopped with dH<sub>2</sub>O and revealed by incubating with 1% (NH<sub>4</sub>)<sub>2</sub>S v/v for exactly 1 min. A control slide in the absence of nucleotide, in which no reaction is expected, is included in the experiment. Nuclei are counterstained with hematoxylin. Samples are then mounted with aqueous mounting medium (e.g., Fluoromount™, Sigma-Aldrich); dehydration is not recommended because of the eventual loss of lead precipitates. Finally, samples are observed and photographed under light microscope; enzyme-active sites are brownish black. An adapted protocol with slight modifications, including the replacement of ammonium sulfide by glutaraldehyde, can be applied for electron microscopy [20]. **Table 1** includes buffer formulations.

PRE-INCUBATION BUFFER			
Reagent	Stock Solution	Final Concentration	Volume (iV=1mL)
Tris-maleate	200 mM	50 mM	250 µL
Sucrose	1 M	0.25 M	250 µL
MgCl <sub>2</sub>	1 M	2 mM	2 µL
Milli-Q H <sub>2</sub> O	/	/	498 µL

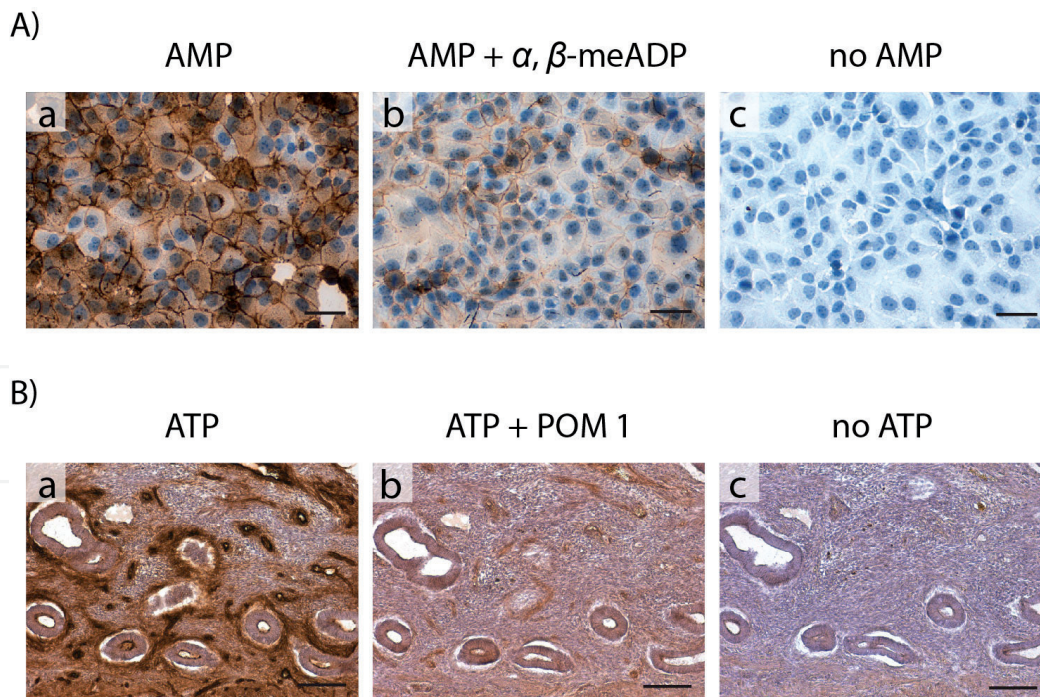
  

ENZYME REACTION BUFFER			
Reagent	Stock Solution	Final Concentration	Volume (iV=1mL)
Tris-maleate	200 mM	50 mM	250 µL
Sucrose	1 M	0.25 M	250 µL
Dextran	10 %	3 %	300 µL
MnCl <sub>2</sub>	1 M	5 mM	5 µL
MgCl <sub>2</sub>	1 M	2 mM	2 µL
CaCl <sub>2</sub>	1 M	2 mM	2 µL
Levamisole	250 mM	2.5 mM	10 µL
Pb(NO <sub>3</sub> ) <sub>2</sub>	1 M	2 mM	2 µL
Substrate	10 mM	1 mM	100 µL
Milli- Q H <sub>2</sub> O	/	/	79 µL

**Table 1.**

*Formulation of buffers used for the lead phosphate-based method. The most right column includes the reagent amounts calculated to prepare a 1 mL final volume (FV) solution. Different nucleotides can be used as substrate. Inhibitors can also be added to both preincubation and reaction buffers; H<sub>2</sub>O to adjust the volume must then be modified accordingly.*

Besides levamisole, enzyme inhibitors might be included in both preincubation and incubation buffers. For example, 1 mM  $\alpha,\beta$ -methylene-ADP efficiently inhibits CD73, and POM 1 inhibits NTPDases (**Figure 3**).



**Figure 3.**  
(A) *In situ* AMPase activity in the endometrial carcinoma human HEC-1B cell line in the presence of 1 mM AMP (a). Note that most of the activity is located at the cell membrane, where CD73 is expressed. Incubation in the presence of the inhibitor  $\alpha,\beta$ -methylene-ADP ( $\alpha,\beta$ -meADP) drastically diminished the activity (b). No AMPase activity is detected when AMP is omitted in the reaction (c). (B) *In situ* enzyme ATPase activity in human endometrium in the presence of 1 mM ATP (a). Activity is strongly inhibited with the NTPDase inhibitor POM 1 (b). No ATP activity is detected when ATP is omitted (c). Scale bars are 50  $\mu\text{m}$  (a) and 200  $\mu\text{m}$  (B).

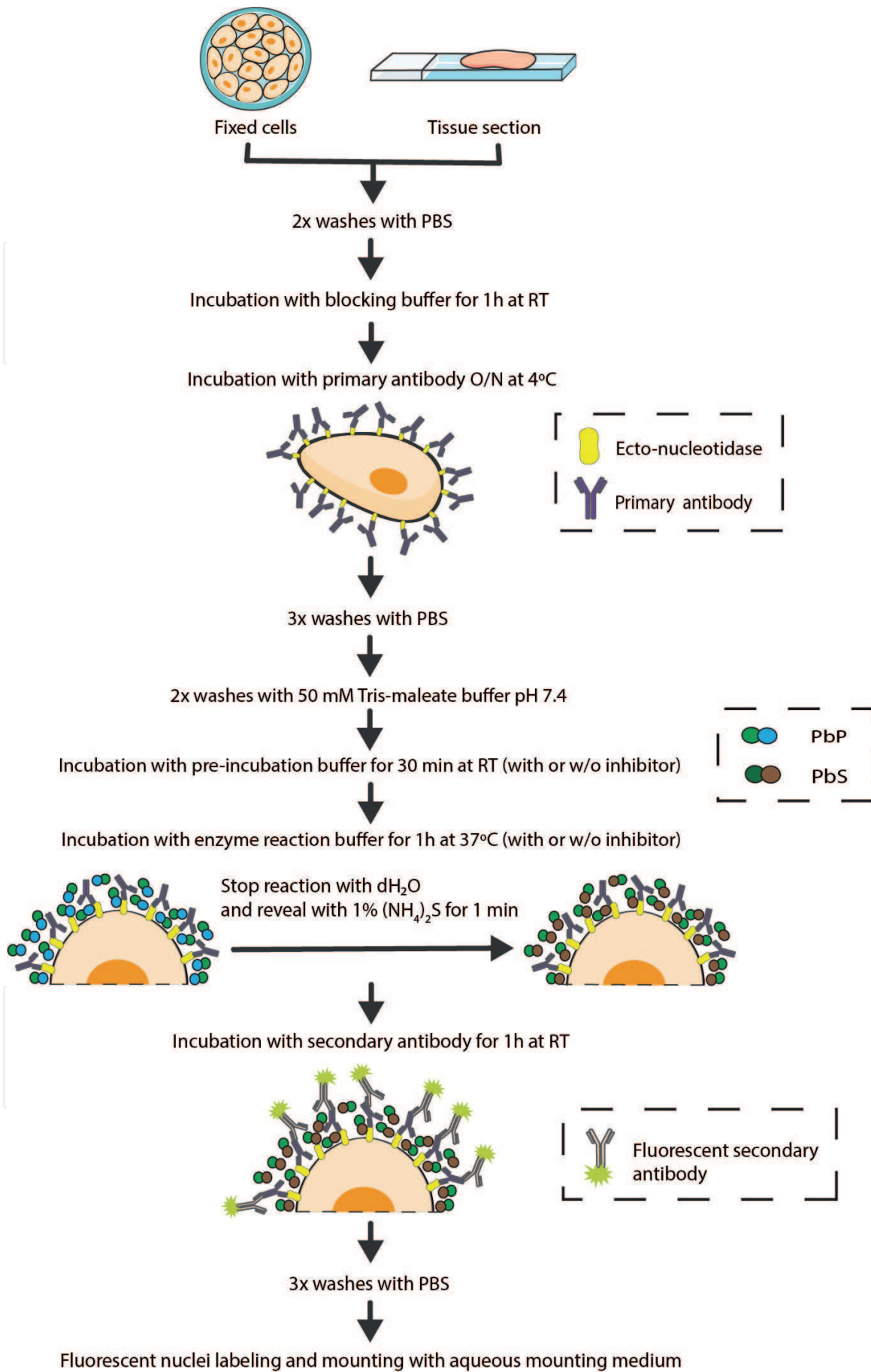
### 2.1.2 In cell cultures

Cells are seeded onto coverslips and allowed to grow with their regular medium until the desired confluence is achieved. The medium is then removed, and the cells are washed twice with PBS before fixation with 4% paraformaldehyde for 5–10 min at RT. Cells are washed three times with PBS to wash out the fixative and kept at 4°C with PBS until use. To proceed with the protocol, coverslips are washed twice for 5 min with gentle rocking with 50 mM Tris-maleate buffer pH 7.4 at RT and then incubated for 30 min at RT with the preincubation buffer. The following steps are as reported for the tissue slices.

### 2.2 Gossrau-based method for APs

*In situ* localization of APs can be addressed by using the Gossrau method that utilizes nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as artificial substrates for the APs. Briefly, tissue slices or fixed cells grown on coverslips are washed twice in 0.1 M Tris-HCl buffer pH 7.4 containing 5 mM  $\text{MgCl}_2$  and then preincubated with the same buffer at pH 9.4 for 15 min at RT. Enzymatic reaction starts by adding the revealing reagent BCIP/NBT (Sigma-Aldrich) for 7 min (up to 15 min) at RT and stopped with 0.1 M Tris-HCl buffer pH 7.4. In AP inhibition experiments, 5 mM levamisole can be added to both preincubation and enzyme reaction buffers. In control experiments, the revealing reagent is omitted. Since the reaction generates blue precipitates, nuclei staining with hematoxylin should be avoided. Alternatively nuclei can be counterstained with methyl green dye. Samples are then mounted with aqueous mounting medium (e.g., Fluoromount™, Sigma-Aldrich) and observed and photographed under light microscope.





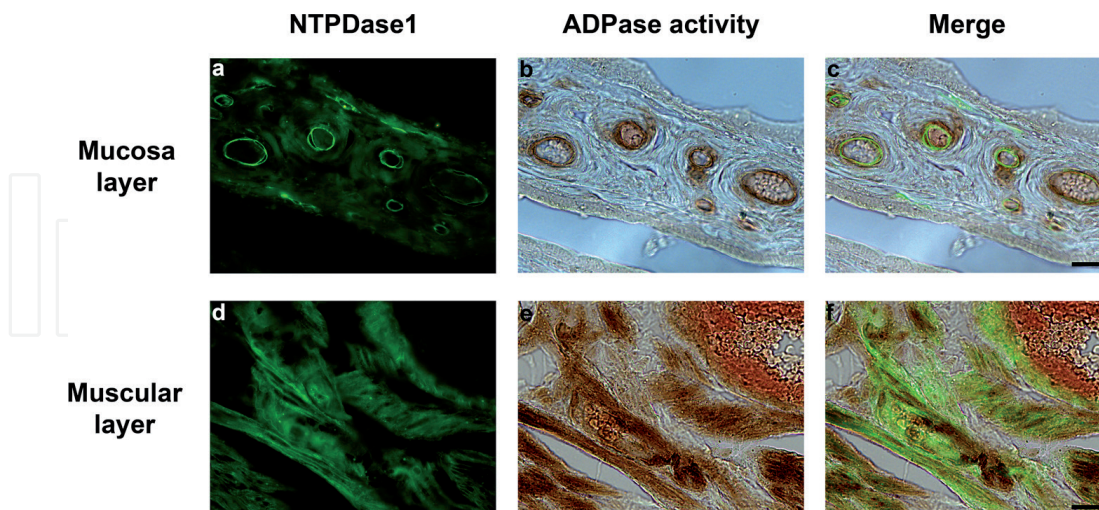
**Figure 4.** Scheme including the main steps of the method combining immunofluorescence and in situ ecto-nucleotidase activity detection protocols.

### 2.3 Combined immunolabeling and in situ nucleotidase activity experiments

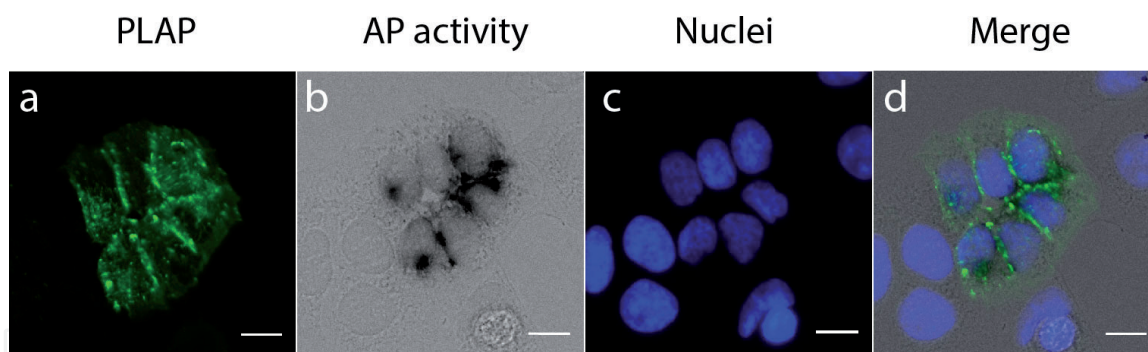
The technique uses the same tissue slide (or the same coverslip of cells) to identify both the activity, with in situ histochemistry (or cytochemistry), and the protein, with immunofluorescence (**Figure 4**) [21, 22]. Tissue sample sections or fixed cells grown on coverslips are washed twice with PBS and incubated with a blocking solution containing 20% normal goat serum and 0.2% gelatin in PBS at RT for 1 h and then incubated O/N at 4°C with the appropriate primary antibody. The samples are washed three times with PBS and twice with 50 mM Tris-maleate buffer. In situ nucleotidase activity experiment is performed as detailed previously, adding the appropriate nucleotide as substrate. Subsequently, the tissues are washed three times in PBS before incubating with the appropriate fluorescent-labeled secondary antibody. After three final washes with PBS, nuclei are labeled, and the samples mounted with aqueous mounting medium; a mounting medium containing DAPI can be used for this purpose (e.g., ProLong Gold antifade reagent with DAPI mounting medium from Thermo Fisher Scientific). The sections are observed and photographed under a Nikon Eclipse E800 Microscope. Pictures of bright field (for activity) and fluorescence (for protein immunolocalization and nuclei visualization) are taken sequentially from the same field.

We recommend that histochemistry be performed between primary and secondary antibody incubations, but other protocols are also feasible. This is of interest when using inhibitory antibodies. In these cases the in situ histochemistry should be performed at the beginning of the procedure. It also has to be taken into account that it might be necessary to test different nucleotide concentrations and incubation times in order to optimize the results for a particular tissue in order to minimize hampering of fluorescence capture by the dark brown lead deposits.

**Figures 5 and 6** are examples of this combined technique in tissue and cell culture, respectively. **Figure 5** shows immunofluorescence to localize NTPDase1, and in situ histochemistry for the ADPase activity in human fallopian tubes. The



**Figure 5.** Immunolocalization of NTPDase1 (a, d) and in situ ADPase histochemistry (b, e) in cryosections of human oviducts. NTPDase1 was detected with immunofluorescence in endothelial cells of lamina propria (a) and in smooth muscle cells (d). Microphotographs b and e show dark brown deposits corresponding to in situ ADPase activity. Merge images (c, f) confirmed that NTPDase1 is active in the same structures where it immunolocalizes. Reddish structure at top right of image is the blood inside the vessel. Scale bar is 25  $\mu$ m. Reprinted by permission of Springer Nature Histochemistry and Cell Biology, Characterization of Ecto-nucleotidases in Human Oviducts with an Improved Approach Simultaneously Identifying Protein Expression and In Situ Enzyme Activity, Villamonte et al. [21].



**Figure 6.** Placental-like alkaline phosphatase (PLAP) immunofluorescence (a) and *in situ* enzyme AP activity (b) in the Ishikawa endometrial carcinoma cell line. Nuclei are labeled with DAPI (c). Merge image (d) shows that precipitates are formed in cells expressing PLAP. Note that activity microphotograph (b) was obtained in gray scale, and in consequence blue deposits are visualized in black. Scale bar is 25  $\mu\text{m}$ .

antibodies used were mouse antihuman NTPDase1 primary antibody (clone BU-61, Ancell) and Alexa Fluor 488 goat anti-mouse secondary antibody (Thermo Fisher Scientific). Label is seen together with the lead precipitate in endothelium of blood vessels, especially abundant in the lamina propria of the mucosa layer, and in muscle cells, predominant in the muscular layer [21]. In **Figure 6**, the antibody against human placental-like alkaline phosphatase (PLAP; clone 8B6, Sigma-Aldrich) was used in Ishikawa cells to localize the protein by immunofluorescence, together with the activity obtained with the BCIP/NBT reagent.

### 3. Conclusions

In conclusion, *in situ* histochemistry for ecto-nucleotidases is an easy-to-perform, reproducible technique suitable for tissues and cells. The combined technique allows identification of the protein that has a precise enzyme activity. The technique is suitable for testing enzyme inhibitors.

### Acknowledgements

This study was supported by a grant from the Instituto de Salud Carlos III (FIS PI15/00036), co-funded by FEDER funds/European Regional Development Fund (ERDF)—“a Way to Build Europe”—/FONDOS FEDER “una manera de hacer Europa”, and a grant from the Fundación Merck Salud (Ayuda Merck de Investigación 2016-Fertilidad). ARM was awarded a fellowship from the Asociación Española Contra el Cáncer (AECC). We thank CERCA Programme (Generalitat de Catalunya) for the institutional support. We are grateful to Serveis Científics I Tecnològics (Campus Bellvitge, Universitat de Barcelona) for the technical support. The authors thank Tom Yohannan for language editing.

**Figure 5** is reprinted by permission of Springer Nature Histochemistry and Cell Biology, Characterization of ecto-nucleotidases in human oviducts with an improved approach simultaneously identifying protein expression and *in situ* enzyme activity, Villamonte et al. [21]. License number: 4487101229606.

### Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this book chapter.

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