Immunogold labelling to detect SpCas9 in cell culture and tissues by electron microscopy

Running title: Immunogold labeling for SpCas9 detection

María José Ulloa-Navas[1], Patricia García-Tárraga[1], José Manuel García-Verdugo[1], Vicente Herranz-Pérez[1,2]*

[1]Laboratory of Comparative Neurobiology, Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia, CIBERNED, 46980 Paterna, Spain.

[2]Predepartamental Unit of Medicine, Faculty of Health Sciences, University Jaume I, 12071 Castelló de la Plana, Spain.

***Corresponding author:**
Vicente Herranz-Pérez, Ph.D.
Laboratory of Comparative Neurobiology, Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia, CIBERNED
Address: C/ Catedrático José Beltrán Martínez 2, 46980 Paterna (Valencia), Spain.
E-mail: vicente.herranz@uv.es
Telephone: +34 96.354.37.69

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Abstract

CRISPR-Cas9 system is a powerful and yet precise DNA-editing tool in rapid development. By combining immunogold labelling and electron microscopy with the novel CRISPR-Cas9 system, we propose a new method to gain insight into the biology of this tool. In this study we have analyzed different Cas9-induced systems such as HEK293T cell line, murine oligodendrocyte progenitor cells, brain and liver to detect Cas9 expression by immunoelectron microscopy. Our results show that while Cas9 expression could be found in the nuclei and nucleopores of transfected HEK293T cells, in transfected oligodendrocyte precursor cells Cas9 was found in cytoplasmic vesicles. In Cas9 constitutively expressing oligodendrocyte precursors the enzyme was located in the cytoplasm of non-dividing cells. Finally, while in the liver Cas9 was detected in different cell types, in the brain we found no specifically-labeled cells. In conclusion, immunoelectron microscopy opens a new spectrum of opportunities to study the CRISPR-Cas9 system in a more precise manner.

Introduction

Genome editing has become one of the most studied fields among the biomedical sciences in the last five decades. CRISPR-Cas systems have skyrocketed the number of applications in genome engineering.[1] First and foremost, CRISPR-Cas systems were described as an adaptive immune system in bacteria and archaebacteria against viruses and plasmids.[2,3] As an application for eukaryotic cells, CRISPR-Cas9 was originally described as a powerful tool to modify specific genome *loci*.[4] Nonetheless, today CRISPR-Cas systems are not only used to edit the genome and epigenome, but also to target, trace and regulate its expression.[5]

The effectiveness of CRISPR-Cas systems to perform any activity in eukaryotic cells resides in different factors, such as the metabolic state of each cell type, which determines the biosynthesis and availability of the enzyme within the cell, and the stage of the cell cycle in which the enzyme is synthesized.[6–8] Another aspect to take into consideration is the ability of each cell type to transport Cas9 into and out of the nucleus or the target organelle. Finally, the ability of each cell type to degrade Cas9 after a time remains to be elucidated.

Another current topic usually debated in this field is the optimization of the transfection method, and how the CRISPR system *per se* can modify the characteristics of any cell type, especially in the case of stem cells or primary cultures, implying one of the most studied issues among the field.[9–11] Although high transfection efficiencies have been achieved in a wide variety of stem cells,[12–16] a gold standard to analyze not only transfection efficiency as a molecular parameter, but also the fitness status of cells in culture, has not been yet established.

Recently, some CRISPR-Cas applications to target extranuclear DNA in different organelles, such as the mitochondria, have been developed.[17] Furthermore, state-of-the-art technology nowadays allows to direct this system to different compartments within the nucleus.[18] Therefore, it is important to develop a high resolution tool to track Cas9 at a subcellular localization level with high precision.

In the present study we present transmission electron microscopy (TEM) and immunogold (IG) labelling, as a method to analyze the above-mentioned criteria. TEM is a classic technique that presents advantages such as a high definition resolution at a single cell level. When combined with IG labelling, it permits to observe the subcellular location and distribution of a protein of interest.[19,20] Since IG-TEM can be used to study both tissues and cell cultures, it can be considered as a valid tool to detect Cas9 at a single cell or single organelle level and to evaluate the ultrastructural characteristics of modified cells. Therefore, here we extensively describe a method to detect *Streptococcus pyogenes* Cas9 (SpCas9) by IG labelling and TEM detection, both in Cas9-expressing cell cultures (HEK293T cell line and oligodendrocyte precursor cells, OPCs) and mouse tissues (liver and brain) comparing different approaches to deliver the enzyme, such as lipofection and transgenic tools.

Materials and Methods

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich.

HEK293T culture

HEK293T cells (ATCC® CRL-3216™) were seeded at a density of 4.2×10^4 cells/cm^2 in 13 mm glass coverslips in 24 well plates or 8 well Nunc Lab-Tek permanox chamber slides coated with 10 µg/mL laminin. Cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 2 mM L-glutamine, 1X antibiotic-antimycotic solution, 5% fetal bovine serum (FBS; all of them from Gibco) at 37 ºC, 5% CO_2.

OPC isolation

OPCs were isolated from P0 C57BL6/J (Charles River) or P0 B6J.129(Cg)-Igs2tm1.1(CAG-cas9*)Mmw/J, also known as H11-Cas9 (The Jackson Laboratory), mouse brains in order to isolate wild-type or Cas9 endogenously expressing OPCs, respectively. H11-Cas9 mouse line has been previously used for pre- and post-mitotic brain cell edition by Nishiyama and colleagues.[21] The telencephalon was dissected in ice-cold HBSS with Ca^{2+} and Mg^{2+} (Gibco). Brains were transferred to 50 mL Falcon tubes (Fisher) in 10 mL of ice-cold HBSS with Ca^{2+} and Mg^{2+} and subsequently incubated at 37 ºC for 5 minutes in papain solution consisting of: 0.09 mg/mL papain (Worthington), 0.02 mg/mL EDTA (Gibco) in 1X HBSS without Ca^{2+} and Mg^{2+} (Gibco). Tissue was dissociated with sterile scissors and repeated pipetting through 1000 µL pipette tips. The papain was neutralized using a media consisting of 5% FBS and 1X antibiotic-antimycotic solution in DMEM (all from Gibco). The resulting cell suspension was filtered through a 70 µm mesh (BD Biosciences) and centrifuged at 300 *g* for 10 minutes. Then we isolated the OPC fraction using PDGFRα-MACS (Milteny Biotech) as recommended by the manufacturer. Following this, cells were seeded at a density of 5.25×10^5 cells/cm^2 in laminin coated 13 mm glass coverslips or in 8 well permanox chamber slides and cultured in DMEM medium containing 1X N-2 and B-27 supplements (Gibco), 10 ng/µL bFGF and 20 ng/µL PDGF-AA (Peprotech). Medium was replaced the next day and then every other day until cells were subcultured for transfection. This process was reviewed and approved by the Animal Welfare Ethical Review Board of the University of Valencia (2017/VSC/PEA/0017).

Cell culture transfection

For Cas9 expression we used pSpCas9(BB)-2A-GFP plasmid (PX458), a kind gift from Dr. Feng Zhang (Addgene plasmid #48138). For GFP expression we used pmaxGFP (Lonza). Plasmids were purified using GenJet MiniPrep Kit (Thermo Fisher).

OPCs were transfected after 13 days *in vitro* using 750 ng of plasmid with Lipofectamine LTX with Plus reagent (Invitrogen) as recommended by the manufacturer in each well of 24 well plates or with 375 ng in each well of 8 well chamber-slides. HEK293T cells were transfected using 500 ng of plasmid in each well of 24 well plates or 250 ng of plasmid in each well of 8 well chamber-slides, as described by Ran and colleagues,[22] with Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer.

Sample processing for immunofluorescence and immunogold labelling

Cell culture fixation

Cells were rinsed with 0.1 M phosphate buffer (PB) at 37 ºC and fixed with 4% paraformaldehyde (PFA) in 0.1 M PB at 37 ºC for 10 minutes. Then, the cells were further incubated at 4 ºC for 50 minutes. Finally, fixative solution was removed and cells were gently washed with 0.1 M PB.

Tissue fixation and processing

P60 H11-Cas9 mice (n = 6) and P60 C57BL6/J (n = 3) mice were perfused using 0.9% saline solution at 37 ºC for 5 minutes using a flow rate of 4.5 mL/min. Then the animals were fixed by transcardial perfusion with 4% PFA in 0.1 M PB at 37 ºC for 15 minutes. The liver and the brain were extracted and post-fixed in the same solution overnight. This process was reviewed and approved by the Animal Welfare Ethical Review Board of the University of Valencia (2017/VSC/PEA/0017). For immunofluorescence detection (IF), the organs were dehydrated and embedded in paraffin. Following this, 5 µm sections were obtained with an HM 340E microtome (Microm). For immunoelectron detection, 50 µm sections were obtained using a Leica VT1000S vibratome.

Immunofluorescence detection

Cells and sections were incubated in 1:200 Immunosaver (Electron Microscopy Sciences) in water at 60 ºC for 30 minutes. Peroxidase blocking was performed using a solution of 10% methanol and 10% H_2O_2 in 0.1 M PB. For permeabilization, the samples were washed 3 times for 5 minutes in PTA solution: 0.1% Triton X-100, 1 mg/mL bovine serum albumin (BSA) in 0.1 M phosphate buffer saline (PBS). The samples were then incubated in blocking solution (10% Casein, 5% normal goat serum in PTA) for one hour at room temperature. Subsequently, the samples were incubated overnight in primary antibodies diluted in blocking solution (1:300 rabbit-anti-Olig2, Chemikon; 1:100 mouse-anti-SpCas9, Abcam; or 1:1000 rabbit-anti-GFAP for cell culture, DAKO). The following day samples were thoroughly washed in PTA, and incubated in secondary antibody solution (1:500 AlexaFluor 488 goat-anti-mouse, Invitrogen; or 1:500 AlexaFluor 555 goat-anti-rabbit, Invitrogen). Samples were then washed in 0.1M PB and incubated in 1:1000 DAPI in water for 5 minutes. Finally, the samples were mounted with FluorSave (Calbiochem-Millipore).

Immunogold labelling for cell culture

Cell cultures cultured on 8 well permanox chamber slides were permeabilized with 0.1% Triton X-100 in 0.1M PB for 10 minutes. Following this, the cells were incubated in a blocking solution consisting of 0.3% BSAc (Aurion), 0.05% sodium azide in 0.1 M PB for one hour. Subsequently, the cells were incubated in primary antibody (1:50 mouse-anti-SpCas9, Abcam; 1:150 rabbit-anti-Olig2, Millipore; or 1:200 chicken-anti-Olig2, AvesLab) in blocking solution overnight at 4 ºC. The samples were rinsed in 0.1 M PB and then incubated in secondary antibody blocking solution consisting of 0.5% BSAc (Aurion), 0.025% CWFS gelatin (Aurion), 0.05% sodium azide in 0.1M PB for 1 h, followed by incubation in secondary antibody (1:50 goat-anti-mouse IgG gold ultrasmall, Aurion) diluted in the same solution overnight at 4 ºC. To enhance gold labelling, we performed silver enhancement (R-GENT SE-LM, Aurion) for 15 to 25

minutes in the dark, followed by gentle washing in 2% sodium acetate and incubation in gold toning solution (0.05% gold chloride in water) for 10 minutes. The samples were then washed twice with 0.3% sodium thiosulfate in water. Finally we post-fixed with 2% glutaraldehyde (Electron Microscopy Sciences) in 0.1M PB for 30 minutes. Samples were rinsed and kept in 0.1M PB containing 0.05% sodium azide at 4 ºC until resin embedding.

Immunogold labelling for tissues

Processing for tissue was performed in a similar manner as for cell cultures with a few changes. Briefly, the permeabilization step was performed by repeated freeze-thaw cycles. Before performing this process, the sections were cryoprotected in a solution containing 25% saccharose in 0.1 M PB for 30 minutes, after which the sections were immersed in -60 ºC 2-methylbutane and rapidly transferred to a room temperature saccharose solution. This cycle was repeated 3-4 times. Subsequently, tissues were left in 0.1M PB and then incubated in primary antibody blocking solution for 1 h, followed by primary antibody in blocking solution (1:100 mouse-anti-SpCas9, Cell Signalling) for 72 hours at 4 ºC. The rest of the process was identical to that described for cell cultures.

Sample processing for TEM

Samples were embedded in resin as previously described.[20] Briefly, samples were post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences), 7% glucose in 0.1 M PB for 30 min at room temperature, washed in deionized water, and partially dehydrated in 70% ethanol. Afterwards, the samples were contrasted in 2% uranyl acetate (Electron Microscopy Sciences) in 70% ethanol for 2 h at 4 ºC. The samples were further dehydrated and infiltrated in Durcupan ACM epoxy resin at room temperature overnight, and then at 60 ºC for 72 h. Once the resin was cured, immunolabeled sections were selected and cut into ultrathin sections (60-80 nm) using an Ultracut UC6 ultramicrotome (Leica Biosystems). These sections were placed on Formvar-coated single-slot copper grids (Electron Microscopy Sciences) stained with lead citrate and examined at 80 kV on a FEI Tecnai G^2 Spirit (FEI Company) transmission electron microscope equipped with a Morada CCD digital camera (Olympus).

Statistical analysis

Quantitation of transfected OPCs and HEK293T cells was performed in triplicate experiments by counting the number of immunofluorescent positive cells relative to the total number of cells in three different fields (Supplementary Fig. 1A-E). Statistical analyses were performed using Prism 8 (GraphPad). Differences among different time-points were assessed by one-way ANOVA followed by Tukey's post-hoc test. All numerical data were expressed as the mean ± standard error of the mean (SEM). All statistical tests were two-sided, and a *p*-value < 0.05 was considered to be statistically significant.

Results

We have evaluated immunoelectron microscopy as a useful technique to precisely detect Cas9 in different biological systems including cell cultures and tissue sections (Fig. 1). First, to

standardize our method in cell lines we have used transfected human embryonic kidney 293 cell line (HEK293T). In addition, we have explored the expression and immunodetection of Cas9 in OPC primary cultures from the mouse brain. Finally, we used a similar strategy to analyze the liver and brain of transgenic mice which constitutively express SpCas9.

Immunoelectron microscopy improves the visualization of the subcellular distribution of SpCas9 in HEK293T cell line

To identify the strengths and flaws of our method, we first transfected HEK293T cells in order to assess the differences between Cas9 immunofluorescence and immunogold detection (Fig. 1). In every case, immunofluorescence was used as a control to evaluate Cas9 expression at the subcellular level. First, we transfected the cells with PX458 plasmid containing SpCas9 and GFP sequences. Subsequent immunofluorescence detection 24 h after transfection showed that both SpCas9 and GFP were present within the nucleus and in the cytoplasm of the cells (Fig. 2A and Supplementary Fig. 1A; 43.94% ± 2.75 GFP^{+} cells; 40.58% ± 2.99 SpCas9^{+} cells; n = 1,650 cells). However, immunogold labeling displayed a finer localization of the enzyme (Fig. 2B-F). By using this method, we observed that cells expressed SpCas9 protein in the cytoplasm, although we could not detect it inside any particular organelle (Fig. 2C). A small fraction of cells expressed SpCas9 exclusively in the nucleus, but not in the nucleolus (Fig. 2D) and, interestingly enough, immunogold labelling allowed us to clearly detect Cas9 in the boundary of many nuclear pores, suggesting a potential nuclear translocation of this protein (Fig. 2E-F).

Together, these results indicate that immunoelectron microscopy is a valuable tool for gaining insight into the subcellular dynamics of Cas9.

Immunoelectron microscopy is a valid method for tracking SpCas9 in transfected primary cells *in vitro* over time

To track SpCas9 at different times after transfection we introduced PX458 plasmid in OPCs and fixed the cultures after 10 h, 24 h, 72 h and 5 days (Fig. 3A-L). Then, we performed IF and IG-TEM detection to identify the subcellular localization and dynamics of SpCas9 over time. At 10 hours we did not detect SpCas9 neither by IF nor by IG (Fig. 3A-C). This was also the case for GFP after IF detection (Fig. 3A), suggesting that the plasmid was not expressed at the shortest time point analyzed in our study. Interestingly, 24 hours after transfection the label was detected by IF and IG in small cytoplasmic vesicles surrounding the nucleus (Fig. 3D-F) and in the perinuclear space, only detectable by IG (Fig. 3E-F). Intriguingly, at this time point we were able to detect only one cell in which Cas9 presented a nuclear localization (Fig. 4A), indicating that the lack of nuclear label in the majority of the cells is not due to a methodological limitation. IF analysis of the cells 72 hours post-transfection suggested that Cas9 was expressed in specific areas of the cytoplasm (Fig. 3G). We corroborated this observation by IG-TEM, which allowed us to determine that Cas9 compartmentalized into small cytoplasmic vesicles (Fig. 3H-I). To further investigate the role of the vesicles that packed the enzyme in this cell type, we tracked SpCas9 5 days after transfection. At this time point, we could observe by IF that SpCas9 was still present in discrete regions of the cytoplasm, as we observed at 24 h and 72 h (Fig. 3D, G, J). By combining immunogold labelling and TEM we detected that the

enzyme localized to electron-dense lysosome-like structures and also autophagosomes, which displayed mitochondria and membranous structures in their content (Fig. 3K-L).

Quantitative analysis of GFP^+ and $Cas9^+$ cells relative to the total number of cells increased with time until 72 h post-transfection and it was sustained at similar levels 5 days after transfection (Fig. 3M-N). We found statistically significant differences in the number of GFP^+ cells between 10 hours (4.75% ± 0.23%; n = 83 cells) and 72 hours post-transfection (21.20% ± 2.55%; n = 110 cells, $p = 0.0002$); 10 hours and 5 days post-transfection (22.25% ± 1.47%; n = 67 cells, $p = 0.0001$); 24 hours (6.99% ± 0.25%; n = 517 cells) and 72 hours post-transfection ($p = 0.0006$); 24 hours and 5 days post-transfection ($p = 0.0004$) (Fig. 3M). The number of $Cas9^+$ cells also increased in a similar trend. We found significant differences between 10 hours (1.45% ± 1.45; n = 83 cells) and 72 hours post-transfection (16.95% ± 3.14%; n = 110 cells, $p = 0.0013$); 10 hours and 5 days post-transfection (14.90% ± 1.05%; n= 67 cells, $p = 0.0033$), 24 hours (4.98% ± 0.06%; n = 517 cells) and 72 hours post-transfection ($p = 0.0068$); 24 hours and 5 days post-transfection ($p = 0.0194$) (Fig. 3N).

To further elucidate whether the specific packaging of Cas9 was due to a transfection issue, we transfected pmaxGFP (Lonza) into OPCs. This plasmid is used as a control for transfection when using GFP as a fluorescent marker. As this plasmid does not express Cas9, we performed IG to detect GFP. Our results indicate that GFP is extensively expressed in the cytoplasm and is not packaged into vesicles (Fig. 4B-C), supporting the notion that vesicle packaging is a specific feature of Cas9 induced expression in this cell type.

Overall, using IG-TEM provides a detailed view at a single cell and subcellular level, allowing to detect Cas9 even in discrete organelles. Our results also suggest that Cas9 expression via lipofection in OPCs can result in enzyme degradation in lysosomes.

IG-TEM favors the validation of an efficient expression method in primary cultures

One of the main issues reported by CRISPR users is the validation of the expression method in primary and hard to transfect cell cultures. As a proof of principle to endorse that ultrastructural analysis contributes to validate an optimal expression method for this type of cultures, we isolated cells that endogenously express SpCas9 to compare their ultrastructure to that of transfected cultures. For this purpose, we isolated OPCs from P0 H11-Cas9 mice and performed the same detection protocols as for transfected OPCs. In this case, using IF we found that Cas9 is widely expressed throughout the cell cytoplasm (Fig. 4D). Still, IG-TEM allowed us to confirm that cells express Cas9 in the cytoplasm. Strikingly, mitotic cells, even those in cytokinesis, did not express Cas9, suggesting that OPCs undergoing cell division do not express the enzyme (Fig. 4E). Thereby, we corroborated that ultrastructural studies are significant to ascertain the basic biology of different cells expressing the CRISPR-Cas9 system.

Immunogold enhances specificity of label detection in the liver

To set up the methodology for Cas9 immunogold detection and TEM processing, we performed IF and IG-TEM on liver and brain tissue of P60 H11-Cas9 mice (n = 6).

First, we used IF to study the expression of SpCas9 by astrocytes and oligodendrocyte progenitors within the motor cortex of the brain (Fig. 5). In the case of Olig2 positive OPCs, Cas9 was expressed within the cytoplasm. This result is consistent with our results in OPC cultures *in vitro*, indicating that Cas9 is in most of the cases in the cytoplasm in this specific cell type (Fig. 5E-H). To identify astrocytes, we used immunolabeling for the glial fibrillary acidic protein (GFAP) together with Cas9 immunolabeling. Using this approach we determined that astrocytes do not express Cas9 in this animal model (Fig. 5I-L). Interestingly, we found Cas9 positive cells with neuronal morphology in the cortex of the brain, with both cytoplasmic and nuclear signal. On the contrary, using immunoelectron microscopy we were not able to detect Cas9 expression in any brain structure after trying several different conditions.

IF of the liver displayed a wide distribution of the label across the tissue (Fig. 6A-D). Intriguingly, immunogold in the liver allowed us to detect the gold labeling broadly distributed in the cytoplasm of some hepatocytes and Kupffer cells (Fig. 6E-H). This is an interesting fact given that the liver is a highly auto-fluorescent organ, and immunogold can be a tool to discard autofluorescence while providing a high-resolution image.

The fact that IG can efficiently detect Cas9 in liver but not brain tissue, suggests that not all tissues may be optimal for immunogold processing and analysis.

Discussion

Cutting edge studies have transformed the bacterial defense system CRISPR-Cas into a potent tool for genome editing,[3,23,24] not only for nuclear but also for extranuclear DNA.[2,4,17,25,26] However, to our knowledge there are no studies that present ultrastructural data for CRISPR applications. Conventional techniques, such as immunofluorescence Cas9 detection, do not allow for subcellular tracking, nor provide super-resolution images that facilitate the understanding of the dynamics of this bacterial enzyme within the eukaryotic cell.

Therefore, the purpose of this study was to develop a tool to achieve Cas9 identification and high-resolution imaging for individual cells. Accordingly, we used different *in vitro* and *in vivo* studies to assay immunogold combined with TEM in order to analyze different cellular features of Cas9 expressing cells.

We transfected HEK293T with PX458 Cas9 expressing plasmid as a control and proof of principle for cell line experimentation. In this aspect, our results using IF show that 24 hours after transfection these cells display a broad distribution of the enzyme throughout the nucleus and cytoplasm. Still, immunogold provides a better resolution for subcellular localization. TEM analysis allows the detection of the enzyme within the nucleus but not in the nucleolus and, furthermore, it permits the visualization of Cas9 in the proximity of nuclear pores, suggesting its nuclear translocation.

As a proof of concept for Cas9 detection in primary cell cultures, we used oligodendrocyte progenitors isolated from P0 mice from control and transgenic animals that constitutively express Cas9. TEM analysis allowed us to detect Cas9 mostly in vesicles and in the perinuclear space, while this effect was not observed when these cells were transfected with GFP. However, recent articles achieve genome editing in OPCs by either using human induced pluripotent stem cells (iPSCs) together with lipofection[27] or by using viral vectors in primary OPCs[28]. This indicates that the method of isolating or inducing OPC production and the transfection method is rather important in this cell lineage. Our results suggest that nuclear internalization and/or enzyme availability in the nucleus of OPCs is scarce and therefore undetectable, opening the field to more studies that take into account aspects to understand and correlate the efficiency of genomic edition with nuclear availability and nuclear transportation in primary oligodendroglial lineage cell cultures.

Taking into account the broad cytoplasmic distribution of GFP in PX458 and pMAX GFP transfections together with our quantitative analysis showing and increase in GFP expression over time, we suggest that GFP is a stable protein in OPCs. We found a similar increase in the number of Cas9 positive cells with time, nevertheless the subcellular distribution of this protein appeared to be restricted to the perinuclear space and cytoplasmic vesicles at short and medium times after transfection (24 h and 72 h respectively). Furthermore, at longer times (5 days after transfection) Cas9 appeared mostly confined to lysosomal vesicles and autophagosomes, suggesting that the enzyme is degraded by OPCs after lipofection. In contrast, Cas9-expressing OPCs displayed a wide cytoplasmic distribution of the enzyme in non-dividing cells. These findings reveal the potential of immunoelectron microscopy to provide complementary information to that offered by other techniques, such as immunofluorescence or western blot, to elucidate the final fate of proteins at the subcellular level. Additionally, IG-TEM favors the comparison of transfected versus non-transfected and dividing versus non-dividing cells within the same culture.

Finally, we also demonstrate that IG-TEM can be successfully used for Cas9 detection in mouse tissues such as the liver. Conversely, while IF was able to reveal Cas9 expression in the brain, IG-TEM was no effective for this purpose. While we cannot rule out the possibility that enhanced methods could improve Cas9 detection in difficult tissues, there is evidence supporting the fact that antibody-antigen interactions can be complex and unpredictable, especially in applications such as immunoelectron microscopy.[29] Technical improvements in antibody design, tissue processing and Cas9 expression will help to overcome these limitations.

Conclusion

In conclusion, we have adapted a classic method such as TEM to a state-of-the-art tool, such as the CRISPR-Cas system, to potentiate this technique in the cell biology field and give a clearer vision and a more exact validation for CRISPR users. Ultrastructural studies together with immunogold labelling contribute to a deeper comprehension of Cas9 biology at the

subcellular level. Nevertheless, further research is needed in this field to better understand CRISPR-Cas9 dynamics in a wider spectrum of cell types and tissues.

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