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Author(s)	Chan, WY; Tam, WY; Yung, KM; Cheung, CS; Sham, MH; Copp, AJ
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Tracking Down the Migration of Mouse Neural Crest Cells

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Wood Yee Chan[a] Wing Yip Tam[a] Kim Ming Yung[a] Chui Shan Cheung[a]
Mai Har Sham[b] Andrew J. Copp[c]

[a]Department of Anatomy, Faculty of Medicine, The Chinese University of Hong Kong, [b]Department of Biochemistry, The University of Hong Kong, Hong Kong, China; [c]Neural Development Unit, Institute of Child Health, University College London, London, UK

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Cell markers · Wheat germ agglutinin-gold conjugate · DiI · *Hoxb2-lacZ* · Green fluorescent protein · Microinjection · Electroporation · Whole embryo culture · Mouse embryo

Abstract
During early embryonic development, cell migration is one of the most important morphogenetic processes. Neural crest cells arise from the dorsal part of the neural tube and migrate along different pathways to numerous locations where they differentiate into a variety of tissues. In the mouse, studies of neural crest cell migration have been difficult partly because of the absence of specific markers which can label neural crest cells throughout their migration from their origin to the site of differentiation. Nevertheless, the use of different experimental strategies involving extrinsic, intrinsic or genetic cell markers has already led to a good understanding of this migration. In our studies, extrinsic markers such as wheat germ agglutinin-gold conjugates and DiI and genetic markers including *Hoxb2-lacZ* and green fluorescent protein have been employed in tracing migrating neural crest cells. The labelling procedures and the strength and weaknesses of the tracing methods are reviewed herein.

Introduction

Cell migration is a major feature of morphogenesis in animals. Cells leave their place of origin and move over long distances along different migratory pathways to their final location where they undergo differentiation. This kind of directed movement of cells from one location to another can be involved in the rearrangement of cell layers, changes in the shape of a developing structure, assignment of cell fate and tissue patterning. Examples of migrating cells during early embryonic development include the haematopoietic stem cells, pigment precursor cells, primordial germ cells and ingressing ectodermal cells through the primitive streak.

During the early development of the central nervous system, cell migration is also a very important morphogenetic process. In humans, from about the 8th week of gestation onwards, postmitotic neuroblasts derived from the

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Wood Yee Chan
Department of Anatomy, Faculty of Medicine
The Chinese University of Hong Kong
Hong Kong (China)
Fax +852 2603 5031, E-Mail wy-chan@cuhk.edu.hk

ventricular zone start their radial migration along radial glial cells to form the cortical plate between the subplate and the marginal zone in an inside-out gradient [1–5]. It has been shown that the pyramidal neurons of the cortex mostly originate in the ventricular zone, whereas the majority of the cortical interneurons appear to be derived from the ganglionic eminence, which is an enlarged structure on the basolateral floor of the lateral ventricle. These interneurons first migrate tangentially through the intermediate zone and then move along the radial glial fibers to reach their cortical locations [6].

Another group of migrating cells in the developing nervous system are the neural crest cells, which originate from the dorsal part of the neural tube. They are capable of migrating over long distances to colonize different regions of the embryo where they give rise to a variety of tissues including dorsal root ganglia, parasympathetic ganglia, adrenal medulla, pigment cells and craniofacial structures [7]. Investigations of migrating neural crest cells in mammals have been difficult, partly because migrating neural crest cells usually do not exhibit special morphological features which can allow them to be distinguished from their neighboring cells and partly because specific cell markers which can label migrating cells throughout their migration from their origin to their final location are not available. This contrasts with the situation in the avian embryo, where use of the technique of quail-chick chimaeras [8] has provided a wealth of information on neural crest migration. Hence, different experimental strategies have been devised to trace migrating cells at different developmental stages of mammalian development.

In this short review, we have no intention of providing an exhaustive summary of tracing strategies, but, instead, we focus on the methods that have been used in our laboratories to track down the migration of neural crest cells in the mouse in the hope that our experience can help to provide hints or clues for designing methods to trace other types of migrating cells.

Extrinsic, Intrinsic and Genetic Cell Markers

Experimental strategies for following cell migration usually require a cell-labelling method to identify the otherwise morphologically indistinguishable cell type. When a cell marker is employed, it should ideally be: (1) cell localized: the marker remains associated with the cell until the cell divides, and following cell division, the marker is passed on to the cell's mitotic descendants; (2) developmentally neutral: the marker does not perturb developmental processes in any way; (3) specific: the marker differentially labels the cell to be followed; (4) stable: the marker is stable during the time period of interest and is not diluted upon cell division; (5) easily and reliably detectable: the marker can be easily visualized in a variety of tissue preparations such as living cells, whole mount tissues and paraffin or frozen sectioned tissues; and (6) compatible with other markers: the marker can be simultaneously visualized with other markers.

For decades, extrinsic cell markers have been used for tracing migrating cells. Vital dyes such as Nile blue sulfate, neutral red and Bismark brown were used as cell markers by direct in situ application [9–11] or grafting an appropriately labelled piece of tissue to an unlabelled recipient [11, 12]. Useful information on neural crest cell migration has also been obtained by using tritiated thymidine to label embryos from which donor tissues were isolated and grafted to unlabelled embryos [13–18]. These cell markers, however, suffer from the problems of dilution as a result of cell division, a lack of specificity due to diffusion to neighbouring unlabelled cells [19] and, in some cases, a certain level of cytotoxicity. Later, more extrinsic markers emerged, which include lectin conjugates (e.g. wheat germ agglutinin-gold conjugates (WGA-Au) [20–23]), carbocyanine dyes (e.g. 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), DiO [24–28]), carboxyfluorescent diacetate (e.g. CFSE [29–33]) and lysinated rhodamine dextran [34, 35]. These markers are more cell localized, give a stronger signal, are easier to detect and are less toxic to cells. In addition, these markers are readily available, easy to prepare for labelling and relatively inexpensive.

Besides the extrinsic markers, the use of intrinsic markers has also been explored for many years. Early studies made use of cytological features such as cytoplasmic inclusions (e.g. pigments, yolk granules), RNA content, cell or nuclear size and differential staining properties [36, 37]. While these markers are useful in identifying clusters of neural crest cells and do not have the dilution problem over cell divisions, difficulties in localizing isolated cells arise when the cells are migrating within the mesenchyme. More recent advances in molecular technology have enabled the use of antibodies or probes to detect expression of neural crest-specific molecules, and these techniques have become the mainstay of studies of mammalian neural crest cell migration. Among the 'expression markers' of neural crest cells, HNK1/NC1 has contributed much to our understanding of the early steps of migration in avian and rat embryos. However, it is not expressed in mouse neural crest cells and is neither a per-

manent nor a specific marker for neural crest cells of other species. HNK1/NC1 is expressed in various other cell types, including the neural tube, perichondrium and heart [38–40]. Other molecules which are expressed by neural crest cells or their derivatives include vimentin-related 4E9R antigens [41], RhoB [42, 43], Pax 3 [44], Sox 10 [45, 46], Hoxa-3 [47], Foxd3 [48], CrabpI [49], Prx1 and 2 [50], c-met [51], MASH1 [52–55], Phox2a [56–58], Phox2b [57–59], AP-2 [60, 61], 5-HT2B receptor [62], tyrosinase [63], receptor tyrosine kinase Ret [64–68], neurotropin receptor $p75^{NTR}$ [53, 69, 70], endothelin receptor B [71] and tyrosine hydroxylase [72–74]. Detection of these molecules has yielded significant information on the migration and development of neural crest cells. The problems associated with these markers are as follows: (1) they may be expressed in non-neural crest tissues; (2) they may not be expressed in all of the migrating neural crest cells and (3) they may not be expressed in neural crest cells, throughout their migration from their origin to their site of differentiation.

Another approach to tracing migrating neural crest cells is genetic labelling. Replication-defective retroviruses carrying the marker gene *lacZ* (which encodes the protein β-galactosidase) have been used as markers by either direct introduction into the neural crest migratory pathway to label the migrating neural crest cells [75] or by infection of neural tube fragments which were then grafted isotopically into uninfected host embryos [76, 77]. Multiple lines of transgenic mice have also been generated that overexpress *lacZ* under the control of different types of promoters or enhancers with the aim of identifying transgenes that are expressed specifically in the neural crest cell population. The dopamine β-hydroxylase-*lacZ* transgenic marker which is expressed by enteric neural crest cells has been used to study the aberrant migration of neural crest cells in mutant mice [78], while a mouse line expressing a *lacZ* reporter in the neural crest cells under the control of the *Wnt-1* enhancer has been employed for studying the defect in neural crest development [79]. Recently, a powerful technique has been introduced, by which neural crest cells are caused to permanently express *lacZ* following Cre-mediated recombination under control of the *Wnt1* promoter [80]. Transgenic embryos which ubiquitously express a marker transgene (e.g. Rosa 26-hPAP and Rosa 26-EGFP [81], X-linked HMG-CoA-*lacZ* [82]) can also be potential sources of labelled cells for the production of chimaeric embryos in studies of neural crest cell migration.

In the following sections, two extrinsic markers, namely WGA-Au and DiI, and two genetic markers, namely a *Hoxb2-lacZ* construct and a green fluorescent protein (GFP) vector, are taken as examples to illustrate how they have been used as markers for neural crest cells.

WGA-Au and DiI

The lectin WGA, with a molecular mass of 35,000, is known to bind to N-acetyl glucosamine and sialic acid residues on the cell surface. After binding to the cell surface, it is quickly engulfed into the cytoplasm by absorptive endocytosis [83]. When tagged with gold particles, WGA can be easily localized intracellularly by electron microscopy or light microscopy using either dark field illumination [20] or following silver enhancement staining, where the gold particles are greatly enlarged by deposition of silver granules on their surface [22, 23, 84]. Alternatively, WGA can also be localized by immunohistochemical methods using an antibody against WGA [85, 86]. WGA is not recycled to the plasma membrane following endocytosis, even though the binding sugar residues may resurface [87], thus minimizing the chance of WGA being transferred to neighboring cells. Ultrastructural studies also show that WGA-Au particles remain within intracellular vacuoles and do not appear in the extracellular space [20, 22]. Studies of double-injected chimaeras, in which two separate populations of labelled neural crest cells, one labelled with WGA-Au and the other with thymidine, were injected into a single embryo, indicate no transfer of markers between populations even when the two populations were mixed together [86, 88]. At the concentration used for labelling, WGA-Au does not perturb the normal development of embryonic cells or other cell types such as neurons [22, 86, 88–92]. However, WGA-Au can only be used as a short-term marker because it becomes diluted in the rapidly dividing neural crest population. We find that the amount of WGA-Au within cells diminishes below detection level 24–48 h after labelling.

Another commonly used extrinsic cell marker is the fluorescent carbocyanine dye DiI. DiI is hydrophobic and lipophilic, and thus easily intercalates into almost all cell membranes that it contacts [93]. Diffusion of DiI from one labelled cell to another appears to be minimal [25, 94, 95]. At the labelling concentration, adverse effects on neural crest development and cellular toxicity usually observed at high DiI concentrations are not evident [25, 95–97]. Furthermore, dilution of DiI over cell divisions is not such a great problem as for WGA-Au, owing to the intense fluorescent signal of DiI.

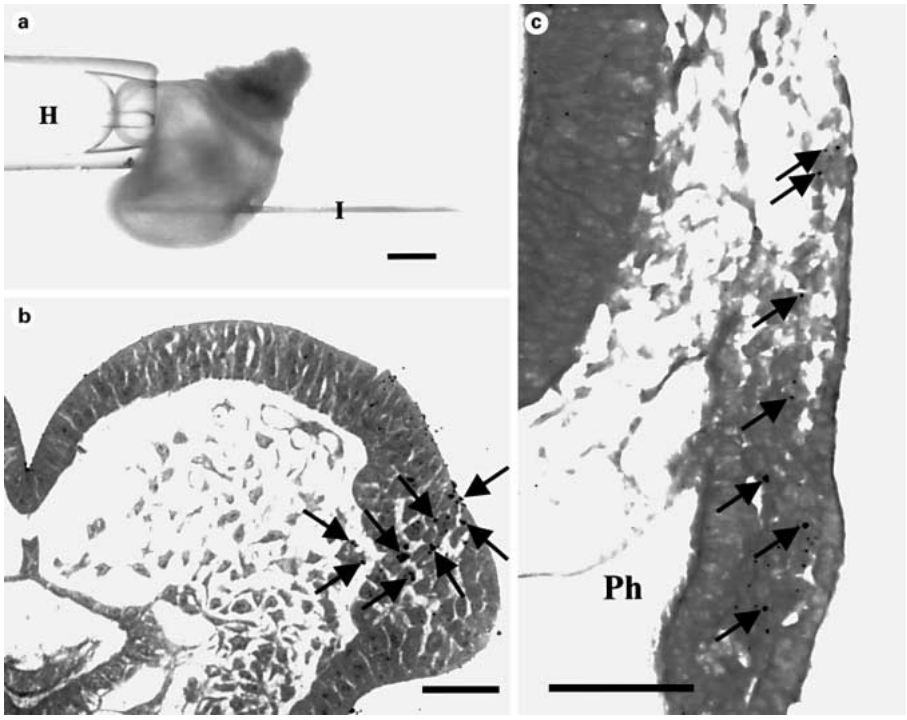

Fig. 1. a Photomicrograph showing labelling of a hindbrain neural crest region with WGA-Au solution using a micromanipulator. A mouse embryo with its yolk sac intact is held by the holding pipette (H) using slight suction, and the injection pipette (I) loaded with the WGA-Au solution (original colour: red) at its tip has been pushed through the yolk sac and amnion to enter the amniotic cavity. A small amount of the WGA-Au solution is being released in the vicinity of the neural crest of the hindbrain region. Bar: 250 μm. b Two hours after labelling, WGA-Au-labelled cells carrying dark intracellular granules (arrows) are found in the neural crest region. Bar: 50 μm. c Twenty-four hours after labelling, WGA-Au-labelled cells (arrows) can be detected migrating in the mesenchyme, while some have already arrived at regions lateral to the developing pharynx (Ph). Bar: 100 μm.

Microinjection and Whole Embryo Culture

Three techniques are commonly used for introducing WGA-Au or DiI into mouse neural crest cells. In blanket labelling, WGA-Au or DiI is injected into the lumen of the neural tube with the aid of a micromanipulator and the dye labels all neural tube cells, including pre-migratory neural crest [25, 95, 98]. A second technique is focal labelling, where a small amount of concentrated WGA-Au or DiI is placed directly to the neural crest region (fig. 1a). With focal labelling, a small population of neural crest cells can be labelled at a selected axial level and cell migration can be followed over time (fig. 1b, c). The third technique is grafting of WGA-Au- or DiI-labelled tissues into unlabelled host embryos. Neural tubes are first isolated from mouse embryos by microdissection following dispase or trypsin-pancreatin digestion [22, 99]. The neural tubes are then labelled with WGA-Au or DiI by immersion in the labelling solution for several minutes before tissue fragments are dissected from the neural crest region. The labelled neural crest fragments are then microinjected into unlabelled host embryos [22], and the migration of the labelled neural crest cells can be followed against the unlabelled background of the host embryo.

As the success rate of re-implanting post-implantation mouse embryos back into the uterus is extremely low [100], the most viable approach is to maintain embryos following labelling in vitro, using the method of whole embryo culture. Embryos whose visceral yolk sac and amnion are intact are grown in rolling bottles containing a culture medium in a thermostatic environment [101, 102]. This in vitro method enables rodent embryos explanted as early as the egg-cylinder stage to develop normally for up to 96 h, during which time the major organ rudiments are formed at a rate comparable to that in vivo [22, 101, 103–105]. The whole embryo culture method has been successfully employed in our laboratories in studies of teratogenic effects of drugs [106–109], limb bud regeneration [110, 111], neurulation [112–114], primordial germ cell migration [115] and neural crest cell migration [22, 23, 116].

Hoxb2-lacZ Construct

Genetic labelling is a powerful approach for tracing the migration of neural crest cells. *Hox* genes are known for their role in specifying regional characteristics along the anterior-posterior axis, and a combination of *Hox* genes

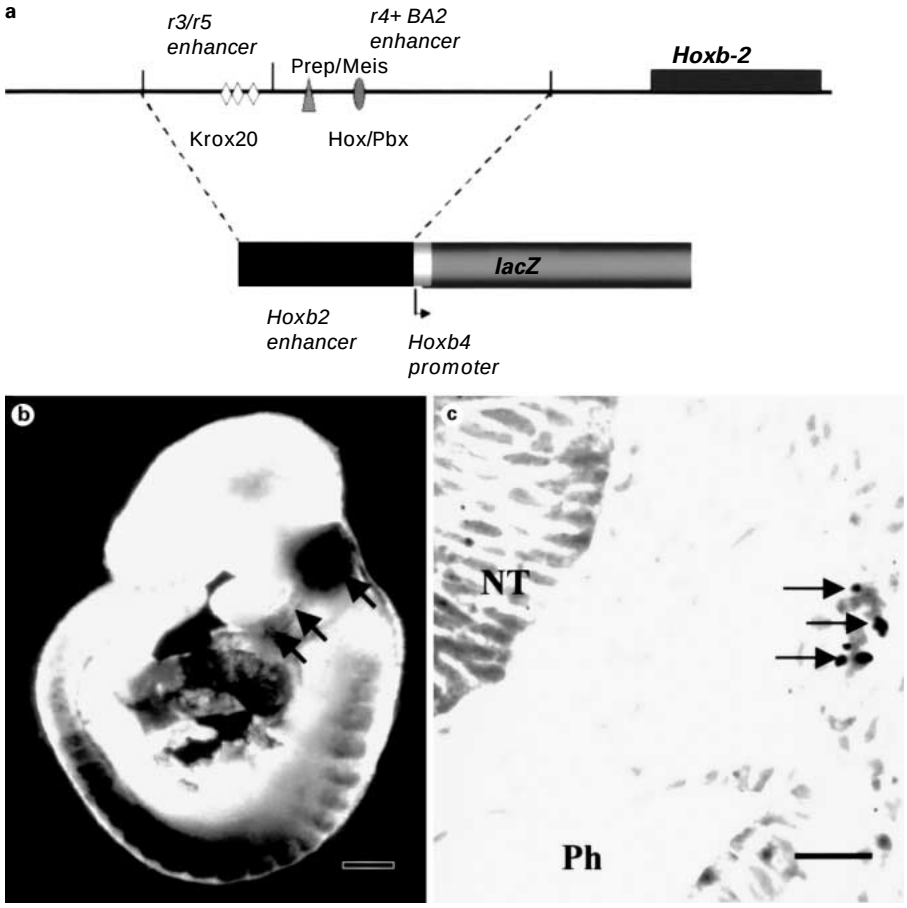

Fig. 2. a Diagram showing the *Hoxb2-lacZ* DNA construct used for generating transgenic embryos [for details of the construct, see ref. 121, 122, 135] [for vector information, see ref. 136]. b A transgenic embryo showing positive cells in the pre-otic hindbrain, cranial mesenchyme and branchial arch regions (arrows). Also note that somites and the heart are also positive. c A transverse section of a transgenic embryo at the pre-otic hindbrain level after WGA-Au labelling. The counter-staining (eosin) has been filtered out during photography and does not show up in this photomicrograph. Hence, only the *lacZ*-expressing cells show positive staining (light black, original colour was blue). Note that WGA-Au-positive cells (dark black, original colour was black) are also *lacZ* positive (arrows). NT = Neural tube; Ph = pharynx. Bar: 500 μm in b and 25 μm in c.

expressed in the various regions of the neural crest specifies aspects of its developmental fate [117–120]. In studies of the regulatory elements that mediate the patterns of Hox gene expression, a *Hoxb2-lacZ* construct (fig. 2a) was used to produce transgenic mouse lines [121, 122]. It was found that *Hoxb2-lacZ* is expressed in the pre-otic hindbrain neural tube and in the neural crest cells migrating from this region towards the branchial arches (fig. 2b). *Hoxb2-lacZ* is not expressed in other regions of the brain, although it is expressed in the somites and developing heart. When the pre-otic hindbrain neural crest of transgenic embryos is labelled with WGA-Au, most of the WGA-Au-labelled neural crest cells also express the transgene (fig. 2c), indicating that the *Hoxb2-lacZ* construct can be potentially used as a marker to specifically label the pre-otic hindbrain neural crest cells.

GFP Vector

GFP was originally introduced as a reporter gene for monitoring the cell-specific control of gene expression and protein localization in both prokaryotic and eukaryotic systems [123]. By microinjection of GFP mRNA into early blastomeres of mouse embryos, GFP was used as a marker for tracing the fate of embryonic stem cells in living mouse embryos [124]. Recently, GFP has been widely used as a reporter in various studies using different techniques for gene transfer [125–131]. Among these techniques, electroporation can generate a unidirectional current which enables targeted delivery of a GFP expression vector to a specific embryonic site [130–133]. Hence, using electroporation, the dorsal regions of the embryo, including the neural crest, can be specifically labelled with a GFP vector (fig. 3a, b). Although the labelling efficacy is only around 40%, successfully labelled embryos exhibit strong GFP signals, which may last for up to 8–10 days [130] and can be easily detected either in living tissues or

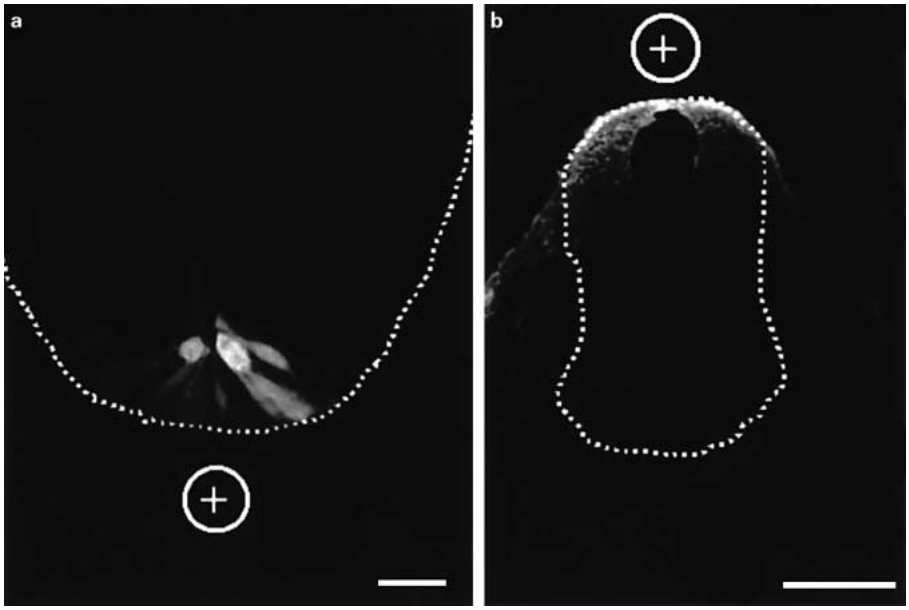

Fig. 3. Photomicrographs showing transverse sections of neural tubes after labelling with GFP by electroporation. Plasmid DNA encoding an enhanced GFP, driven by a CMV promoter, was microinjected into the lumen of the neural tube, and the positive electrode (+) was placed either close to the ventral side (a) or dorsal side (b) of the neural tube (outlined by the dotted line). Note that GFP-positive cells in both paraffin sections (a) and cryostat sections (b) are found on the same side as the positive electrode. Bar: 20 μm in a and 100 μm in b.

in tissue sections using epifluorescence microscopy or confocal microscopy. The GFP signals are well preserved after fixation in 4% paraformaldehyde, during standard procedures for both paraffin (fig. 3a) and cryostat sectioning (fig. 3b) [134]. Therefore, when coupled with whole embryo culture and tissue transplantation, electroporation of a GFP vector into the pre-migratory neural crest can provide an alternative method for tracing the migration of neural crest cells.

Concluding Remarks

An ideal marker to label mammalian neural crest cells throughout their migration has yet to be found, and different experimental strategies have been devised to follow the migration and fate of neural crest cells in different regions of the embryo. Depending on the objectives of the study, appropriate rather than ideal markers have been used. It is hoped that molecular techniques combined with various manipulations such as microinjection, tissue transplantation and whole embryo culture can help to improve our understanding of neural crest development.

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