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RESEARCH ARTICLE

Finding Blue Tracks in *Gephyrocharax melanocheir* Fish Similar to the Locations of Acupuncture Meridians after Injecting Alcian Blue

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Abstract
This study investigated whether a meridian-like distribution of Alcian blue (AB) existed after it was injected into a fish's body and suggested a new animal model for meridian study. Twenty *Gephyrocharax melanocheir* fish with translucent bodies were injected with AB at a point near the spinal column or the dorsal fin. Distribution of AB was observed using a digital camera and a stereomicroscope. Three or more obvious blue tracks were found: one along the spinal column, another along the posterior margin of the abdomen extending to the superior margin of the anal fin, and a third along both sides of the dorsal fin. They were similar to the locations of the governor, conceptual vessel, and urinary bladder meridians, respectively, on the human body according to the classic theory of traditional Chinese medicine. A few other blue tracks were also found, which apparently did not correspond to any known meridians. The results show that the tracks of AB share important similarities with the locations of classically described meridians and that they are mainly distributed in the interstitial space around bones and blood vessels and inside muscular interstices. This study may provide a new experimental animal model for exploring acupuncture meridians.

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1. Introduction

Meridians can be defined as special lines distributed longitudinally on the human body that include sites where acupuncture needling or other forms of stimulation are applied for therapeutic purposes. Their distribution does not coincide, to any significant degree, with any known structures such as blood vessels or nerves. Finding anatomical structures that match the distribution of meridians is, therefore, one of the primary questions in meridian research. For many years, scientists have tried, through various techniques, to prove the existence of the meridians or meridian channels that are described in the classical literature as conduits of "Qi" and blood. In 1963, Bong Han Kim [1] claimed to have found meridian channels and described them as tubal structures, which he named the "Bonghan duct". However, such structures were not confirmed in the research from other laboratories, including those from China [2]. It was not until 2002 that the Korean scientists Shin and Soh [3] and Jiang et al [4] began to study Kim's Bonghan ducts and, using Alcian blue (AB), found novel threadlike structures, seemingly similar to Bonghan ducts, in blood vessels, in lymph vessels, and on the surface of internal organs. They carried out a series of experiments, eventually naming this set of structures the primo vascular system (PVS) [5,6]. However, PVS has not been found on peripheral tissue along the meridians using AB.

Zhang's [7] work has shown that meridians and collaterals are composed of two components: vessels and interstices. Vessels circulate blood, whereas interstices circulate Qi. The latter seems to correspond to interstitial fluid (IF) flow. IF flows in interstitial spaces under the condition of a low hydraulic resistance. Zhang et al [8] have undertaken multiple experiments to prove the existence of low hydraulic resistance channels along classically described meridian pathways. If these are indeed one of the essential characteristics of meridian channels, then this model can also well explain meridian phenomena such as propagated sensation along channels [9], low impedance, and high conduction of sound [10]. These experiments were mostly carried out in mini-pigs, which have been shown to be successful animal models for meridian study. However, channels cannot be observed directly in the body of a live mini-pig, making identification of the underlying structures difficult.

Gephyrocharax melanocheir fish are translucent and small in size. We chose this new animal model to see if it would be possible to observe the AB dye tracks directly inside the body of the fish, to determine whether it would diffuse along the routes of meridians and/or the branch collaterals.

2. Materials and methods

2.1. Animals and reagent preparation

Twenty *G. melanocheir* fish, 4.5 ± 0.5 cm in length and 2.4 ± 0.4 cm in width, were purchased from Beijing Laiguangying fish market and used as experimental animals. They were kept in a fish bowl in water at a temperature of 24°C and given full access to food and oxygen. AB dye (8 GX; Sigma Co., USA) was diluted to 1% and filtered through 0.22-μm pore-sized filter paper. Tricain, a widely used fish anesthetic, was diluted to 0.03 g/L to keep the fish in a quiet state.

2.2. Experimental procedure

The *G. melanocheir* fish was put into 0.03 g/L tricain solution. When the fish body became inclined, it was taken out and fixed on a piece of sponge in a Petri dish. A point near the spinal column or dorsal fin (Fig. 1) was injected using an insulin syringe needle connected to a microinjector at a 45° angle with the fish body to a depth of 1 mm. We injected 20–25 μL AB dye at a speed of 1.2 μL/min, controlled by a microinjection pump (KDS-310-PLUS; KD Scientific, USA). During the injection, the narcosis of the fish was adjusted as necessary with a bottle of tricain solution, which was combined with a bottle of water by a three-way valve. The needle was pulled out after the AB dye diffused completely, and the residual dye was washed away with water. Pictures were taken using a digital camera (D5000; Nikon, Japan) and a stereomicroscope (SMZ1000; Nikon) with the help of a cold light illuminator (Fig. 2).

3. Results

Three or more obvious blue tracks were observed after AB was injected. When AB was injected near the spinal column, a blue track appeared along the spinal column, similar to the location of the governor meridian. In eight fish specimens, blue tracks appeared along the spinal column (Figs. 3A and 3B). The differences in some specimens led us to conclude that AB should be injected close to the spinal column to an appropriate depth. If not, distribution of blue tracks would be short and thick, diffusing in many directions (Figs. 3C and 3D). Note that sometimes the color of the tracks looks green because the fish have a reddish colored body.

When AB was injected near the spinal column, another blue track appeared along the posterior margin of the abdomen, extending to the superior margin of the anal fin. This was similar to the location of the conceptual vessel meridian in humans. Five fish displayed such tracks, which were thin and clear (Figs. 4A and 4B). Using a microscope to observe a small segment of the track, we could see that AB was distributed around bones and it was denser on condyles.

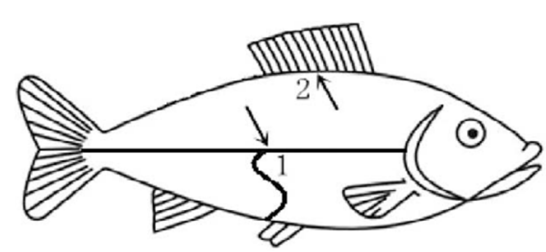

Figure 1 Positions of injection points, as indicated by the arrows.

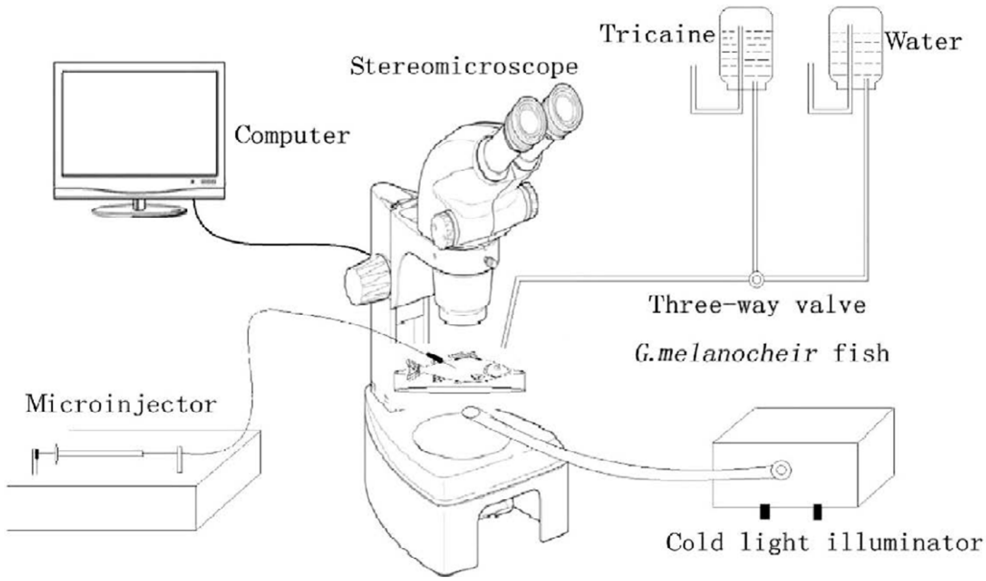

Figure 2 Schematic of the experimental system.

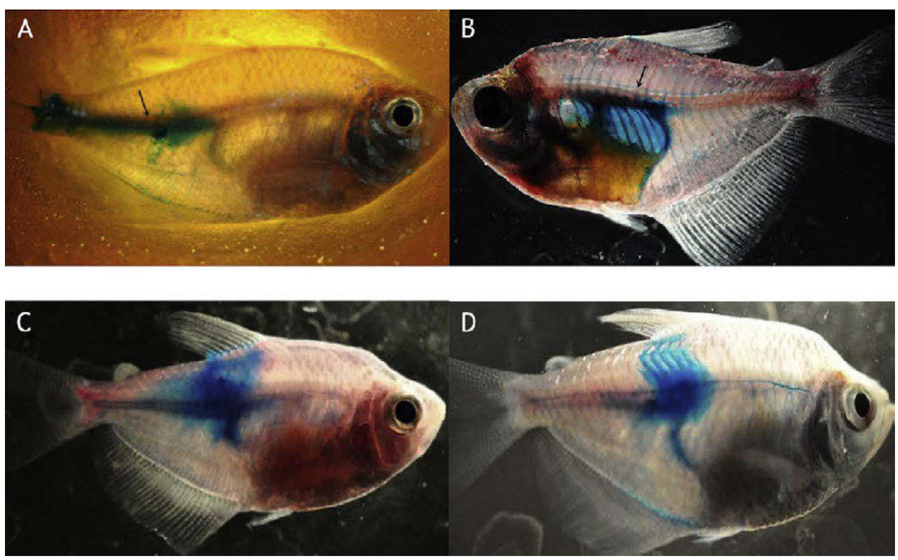

Figure 3 Dye distribution at location similar to the governor meridian. (A) A long zonal blue track along the spinal column. (B) A threadlike blue track along the spinal column. (C, D) Multidirectional diffusion of blue tracks in muscles after injection lateral to the spinal column.

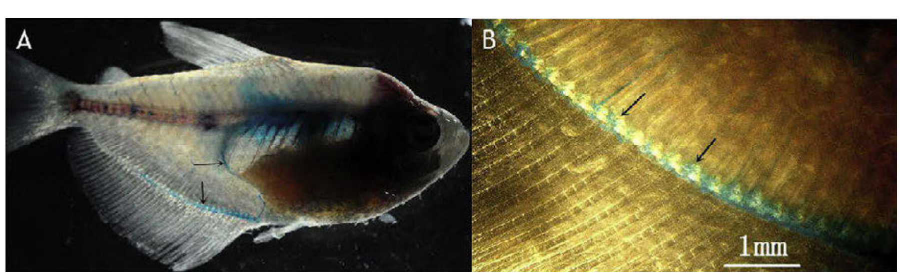

Figure 4 Dye distribution at location similar to the conceptual vessel meridian. (A) A blue track along the posterior margin of the abdomen extending to the superior margin of the anal fin (arrows). (B) A partial picture of Fig. 4A taken by a microscope, showing the distribution of blue track around bones and the density on condyles (arrows).

When AB was injected near the dorsal fin, a third blue track appeared along either side of the dorsal fin (Figs. 5A and 5B), slightly off the midline, and similar to the location of the urinary bladder meridian. Five specimens displayed such tracks.

In some fish, AB distributed along the muscle texture (Figs. 6A and 6B), which is shown in the chart of muscle in fish (Fig. 6C), and branched outward from longitudinal distribution of AB.

We could distinguish that some of the blue tracks were blood vessels (Figs. 7A and 7B). In these cases, the blue color was even and the edges were sharp, indicating the existence of walls that blocked further diffusion of the dye. AB may have entered blood vessels through capillaries.

Sometimes, the dye stayed outside of blood vessels, as seen in Fig. 8. In this case, the red vessel extended from the green vessel, which was thicker than the red one, and the tracks had a blurry edge, indicating that the dye was outside of the blood vessel wall.

Similarly, another blue track might indicate a lymph vessel that was located lateral to the spinal column in the shallow layer under the skin (Fig. 9A). This is in accordance with known fish anatomy (Fig. 9B). The AB might flow from IF into lymph vessels through lymph terminals. Blue-colored branches appeared around the AB area but disappeared beyond the AB region, indicating that there was a flow toward the main trunk inside the vessel.

For the connatural structures, a translucent threadlike sensory organ with an alternating series of nodes could be seen beneath the spinal column (Fig. 10). There is no blue dye on such structures in all the fish.

4. Discussion

4.1. Animal model

Meridians may exist in all living creatures, as they are the basic channels that transport nutrients and energy in living things, according to the theory of traditional Chinese medicine. PVS (described above), which may be one of the primary structures comprising the meridians, has been found in different species [13]. Meridians should therefore also exist in fish, because they are living creatures.

A great advantage of using this kind of fish is that the blue dye can be observed directly in the body of the fish

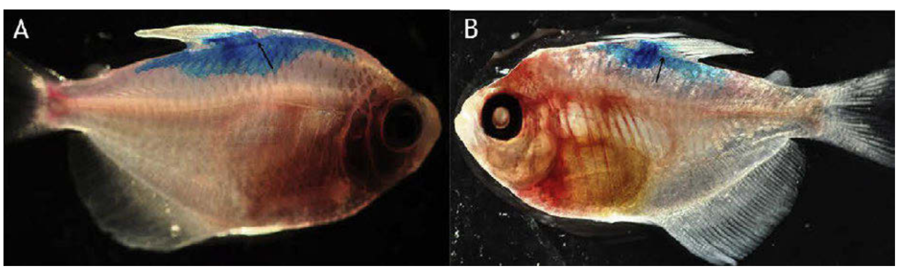

Figure 5 Blue tracks along both sides of the dorsal fin (arrow). (A) The blue track along right side of dorsal fin. (B) The blue track along left side of dorsal fin.

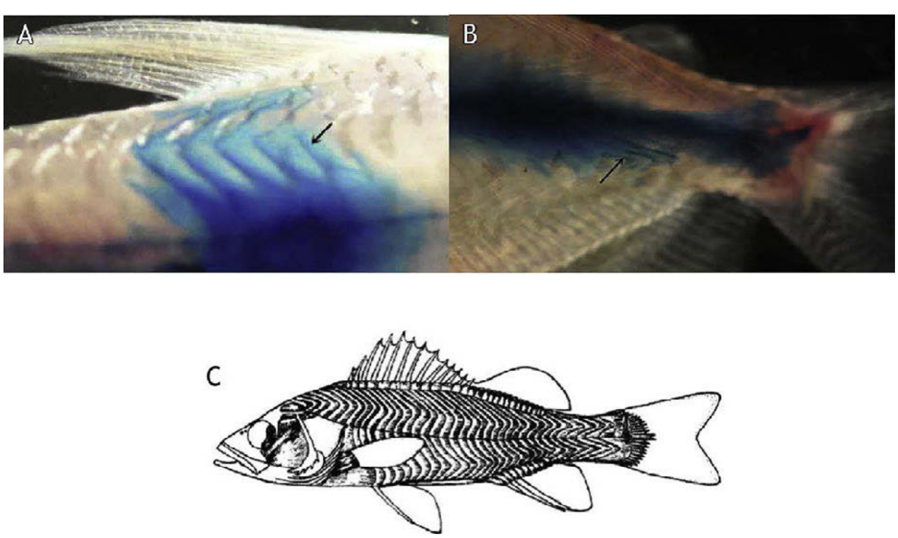

Figure 6 Blue tracks showing muscle texture. (A, B) Parallel distribution of AB along the muscle texture. (C) Sample showing the head and lateral muscles of *Lateolabrax japonicas*, representing muscle distribution of Osteichthyes. *Note*. Figure taken with permission from Meng et al [11]. AB = Alcian blue.

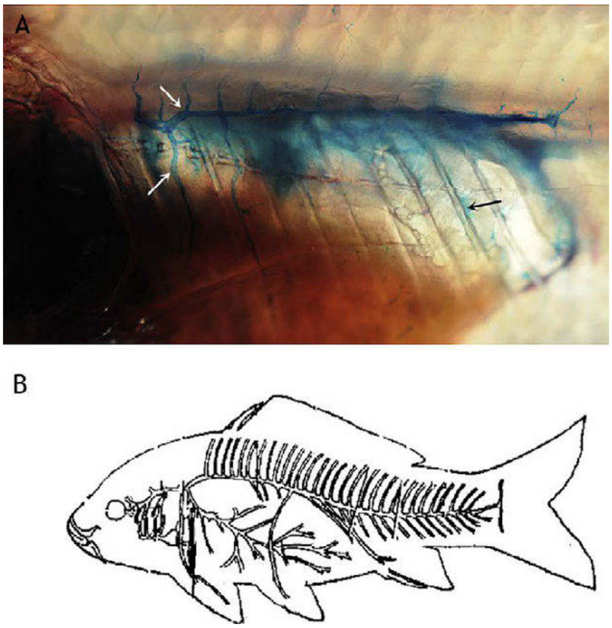

Figure 7 Dye distribution in blood vessels. (A) White arrows indicating blue tracks in blood vessels and black arrow indicating a blue track at the edge of the ribs. (B) Artery distribution of *Cyprinus carpio*, representing Osteichthyes. *Note*. Figure taken with permission from Meng et al [12].

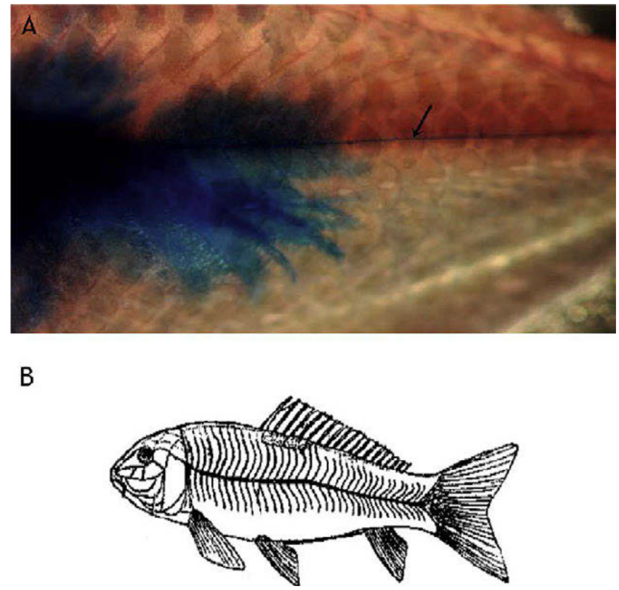

Figure 9 Dye distribution in lymph vessel. (A) A blue track suspected to indicate a lymph vessel located lateral to the spinal column in the shallow layer (arrow). (B) Lymph vessel distribution of *Cyprinus carpio*, representing Osteichthyes. *Note*. Figure taken with permission from Meng et al [12].

because it is translucent, eliminating the need for killing the fish to perform dissection. This is both a technical advantage and a more humane alternative to using pigs or rats as the animal model. In the meantime, some known structures such as bones, blood vessels, muscles, and even lymph vessels could be seen without staining, so that the basic relationships between the threadlike and known structures could be observed. However, only the injected isotope could be seen with a γ-camera using isotopic tracing [14] or positron emission computed tomography experiment of meridians [15]. Therefore, it is still difficult to determine the underlying anatomical structures.

The second advantage is that this kind of fish is available at a very low price, and therefore it is easy to collect a lot of samples, compared to pigs that we have used before, which are relatively expensive. It is also easier to insert a needle into the body of a fish than into the body of a rat or pig. Fixation and suitable anesthesia are also important to get results, because it is difficult to inject the dye and take photographs when the fish is moving. Illumination is important in order to obtain clear photos. To enhance the contrast of AB dye, pink or nearly colorless *G. melanocheir* fish were chosen and the illumination lamps were filtered with blue paper.

The third advantage is the small-sized body of the fish, which makes it convenient to observe the distribution of the dye in the entire body. In contrast, similar experiments in the human body can show only part of the migration of a tracer [16].

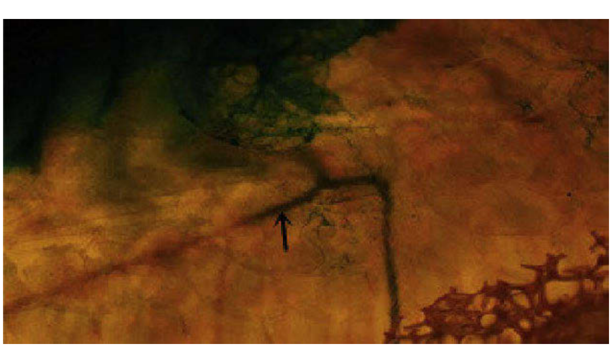

Figure 8 Blue track outside of a blood vessel (indicated by arrow).

Figure 10 A lateral line, which is a translucent tube with an alternating series of nodes, can be observed beneath the spinal column. This sensory organ contains no blue dye (arrow).

4.2. Finding blue tracks

Several blue tracks were found in the bodies of the fish. Most tracks were along the anteroposterior axis, which are similar to the orientation of meridians described in Chinese medicine. The distribution of AB indicates that pathways of flow exist. If there were no specific paths, the AB dye should diffuse in all directions. In the cases shown in Figs. 3C and 3D, the dye indeed diffused evenly, indicating that the injection might not have entered the pathways of flow. Because no meridian chart for fish exists, and it is quite different from humans, we can only evaluate the tracks according to their relative anatomic position. There were three kinds of lines, which are basically coincident with the governor meridian, conceptual vessel, and urinary bladder meridians.

It is difficult to explain these tracks by reference to any known structure except meridians. Both fish and humans are vertebrates, meaning that they have symmetrical bodies and spinal columns; therefore, it was not entirely surprising to find similar meridian-like tracks. Very few people have studied meridians in fish. In our institute, Jin et al [17] have conducted one such study. They stained zebrafish by submerging the fish in 4-(4-diethylamino styryl)-N-methylpyridinium iodide water and found meridian-like sensory organs in the bodies of the fish. However, we concluded that the blue tracks that we observed do not correspond to any sensory organs. The sensory organ could be seen under the spinal column in some fish and it was not stained blue at all (Fig. 9A). The difference is most likely due to the fact that 4-(4-diethylamino styryl)-N-methylpyridinium iodide is sensitive to sensory organs, whereas AB does not specifically stain the organs.

4.3. Location of blue tracks

What exactly are these blue tracks? AB is a dye that can combine with hyaluronic acid, which is the main component of IF. Therefore, the distribution of AB may partly represent that of IF. For the track similar to the governor meridian, the AB dye distributed around the spinal column and its thickness was almost equivalent to that of the spinal column. According to other pictures such as Fig. 7A, the dye might distribute in the gaps between bones and muscles. In the case shown in Fig. 4B, the track is thin and obviously along the interstices of muscles where bone structures have been shown clearly. The AB track outside of the blood vessel in Fig. 8 is quite similar to one of the locations of the PVS and may be related to them [18]. Although AB can specially combine with hyaluronic acid, the blue dye can diffuse only into areas where the dynamics allow the flow or diffusion of injected AB. The path of AB could be low hydraulic resistance channels with a high diffusion coefficient, which exactly represent the main feature of interstices. We can conclude that AB is not randomly distributed in the body of the fish.

4.4. Function of interstices where blue tracks were located

Interstices or interstitial spaces are widely distributed throughout the body and exist in all tissues and organs. These were once thought to be merely the places between different tissues comprising large and small junctions that have no special functions. Interstices are, in fact, not empty spaces, but are filled with extracellular matrix including various fibers, ground substances, and free fluid, which are relatively soft in terms of biomechanics. Most acupoints are located at such "soft spots," which led the famous acupuncturist J.S. Yang to describe them as "San-bian" and "Sanjian" [19].

Some researchers have shown that interstices are closely related to meridians. Zhang [7] pointed out that interstices are actually Qi channels that can be distinguished from Xue (blood) channels. The latter are more similar to blood vessels, according to the theories in the Yellow Emperor's Inner Canon (Huangdi Neijing). Locations of several large interstices are quite similar to the classically described meridians, according to the research on anatomical structures by Xie et al [20]. Our work on fish seems to support these conclusions. The only thing left is to elucidate the functions of interstices, specifically to explore whether there is an overlap between their biological functions and the functions of Qi in traditional Chinese medicine.

Some people suggest that Qi is an electromagnetic wave or a kind of vital energy related to DNA and existing in the PVS [21]. This definition of Qi is more closely related to Source Qi (Yuan-Qi), whereas the Qi running through meridian channels corresponds more to Defense Qi (Wei-Qi), which has the functions of "nourishing tissues and smoothing joints." Broadly speaking, IF in the interstices has exactly these two functions: transfer of nourishing materials from the blood to cells takes place through IF, and smooth fluids such as synovial joint fluid are types of IF. In fact, IF plays a role in smooth gliding of all tissues during the movement of a limb or any other body parts. Fluid movements of Tai Chi (Taiji chuan) perhaps best illustrate this fluid motion. The flow of IF has only recently been recognized by Aukland and Reed [22] and Wiig and Swartz [23]. This important feature of IF enables us to correlate the Qi described in traditional Chinese medicine with IF flow in physiology and apply them to the explanation of traditional Chinese medicine. A typical example is Zhang et al's [24] general illustration of the mechanisms for therapies using the model of IF flow from a clinical perspective. Their study and other studies provide further evidence that IF flow in interstices may be the primary physical structure of meridians and collaterals.

Disclosure statement

The authors declare that they have no conflicts of interest and no financial interests related to the material in this manuscript.

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