Video Article

Intravital Microscopy and Thrombus Induction in the Earlobe of a Hairless Mouse

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Abstract

Thrombotic complications of vascular diseases are one leading cause of morbidity and mortality in industrial nations. Due to the complex interactions between cellular and non-cellular blood components during thrombus formation, reliable studies of the physiology and pathophysiology of thrombosis can only be performed *in vivo*. Therefore, this article presents an ear model in hairless mice and focuses on the *in vivo* analysis of microcirculation, thrombus formation, and thrombus evolution. By using intravital fluorescence microscopy and the intravenous (iv) application of the respective fluorescent dyes, a repetitive analysis of microcirculation in the auricle can easily be performed, without the need for surgical preparation. Furthermore, this model can be adapted for *in vivo* studies of different issues, including wound healing, reperfusion injury, or angiogenesis. In summary, the ear of hairless mice is an ideal model for the *in vivo* study of skin microcirculation in physiological or pathophysiological conditions and for the evaluation of its reaction to different systemic or topical treatments.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55174/>

Introduction

The purpose of the present article is to describe the technique of intravital microscopy applied to the auricle of the hairless mouse for the direct observation and analysis of microcirculation, thrombus formation, and thrombus evolution. With an incidence rate of 1 in 1,000, venous thrombosis is still a common cause of morbidity. Although diagnostics, prevention strategies, and therapies have been developed in recent years, one-third of venous thrombosis manifests as a pulmonary embolism¹. Arterial thrombosis plays a critical role in cardiovascular diseases, which are the most common cause of death in industrial nations. Arterial thrombosis based on the rupture of atherosclerotic plaques is involved in heart attacks, mesenteric infarctions, and apoplexy. Every surgery exposes subendothelial structures to blood components, changes the dynamics of blood flow, and immobilizes the patient. In endoprosthetic surgery of the lower limb, organ transplantation and flap surgery thrombosis are frequent causes of complications. Microvascular thrombosis in particular frequently causes irreversible damage, due to the lack of clinical symptoms. Likewise, microvascular thrombosis plays a crucial role in several diseases, including thrombotic thrombocytopenic purpura, sepsis, disseminated intravascular coagulation, antiphospholipid syndrome, and chronic venous insufficiency, among others.

Several new drugs for the therapy and prevention of thrombosis were developed in recent years, but antiplatelet drugs and anticoagulants still have side effects, lack antagonists, and feature long duration effects. These deficiencies lead to problems in emergency medical care. Thus, more research is needed to uncover the complex processes that occur during thrombosis, which can hardly be simulated *in vitro*.

The hairless SKH1-Hr^{hr} mouse was discovered 1926 in a zoo in London. Due to a gene defect on chromosome 14, the animal loses its fur after postnatal day 10. This makes the well-vascularized auricle accessible to intravital microscopy of the vessels. The average thickness of the ear is 300 µm. It consists of two layers of dermis, which are separated by cartilage. On the convex dorsal side of the cartilage, 3 vascular bundles enter the earlobe. Apical vascular arcs and basal shunts connect the three bundles. The venules have diameters between 200 µm (basal) and 10 µm (apical). Close-meshed capillaries surround the empty hair follicles². The anatomy of the hairless SKH1-Hr^{hr} mouse makes the auricle a powerful and cost-effective model for thrombosis research.

Protocol

All *in vivo* experiments (7221.3-1-006/15) were conducted in accordance with the German legislation on the protection of animals and the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council).

1. General Keeping of the Animals

1. Perform the experiments with male SKH1-Hr^{hr} mice aged 4 to 6 weeks. Use animals with a weight between 20 and 25 g.
2. Keep the animals in a pathogen-free facility and under standardized conditions of 24 to 26 °C and about 60% relative humidity, with steady access to water and food *ad libitum*.
3. Keep up to five male animals in one cage. Provide bedding and enrichment material during the housing of the animals for their well-being.

2. Prearrangement of the Animals

1. Weigh a mouse and load the respective drug (e.g., the cannabinoid, 5 mg/kg bodyweight (bw)) into an insulin syringe. Administer the drug 30 min prior to thrombus induction.
2. By holding the neck of the mouse between the thumb and the index finger and the tail of the mouse with the little finger, stretch the animal and inject the drug intraperitoneally (ip) into the bottom left quadrant of the abdomen. Put the animal back into the cage for 15 min.
3. Prepare anesthesia with ketamine (90 mg/kg bw) and xylazine (25 mg/kg bw). 15 min prior to thrombus induction, anesthetize the mouse. Put the mouse in the cage, pull the tail slightly, and inject the anesthetics ip with an insulin syringe.
4. Put the mouse back into the cage until the onset of anesthesia. To verify sufficient anesthesia, pinch the tail with forceps.
5. Load 0.05 mL of defrosted fluorescein isothiocyanate-labeled dextran (FITC-dextran; 5%, 150 kDa) into an insulin syringe. While filling the syringe, ensure that no air bubbles remain, because even small intravenously (iv)-administered air bubbles can be lethal for the animal.
6. Place the anesthetized mouse on a heating plate in the facedown position. Adjust the heating plate to 37 °C.
7. Put eye ointment on the cornea of the mouse. Disinfect the skin and use sterile instruments.
8. Stitch two sutures of polypropylene 7/0 into the cranial and caudal edge of the right ear. Place the stitches as close to the edge and as proximal to the base as possible (**Figure 1B**).
9. Shift the mouse to dorsal position. Fix all legs to the acrylic glass platform using adhesive strips. Hook a suture under the front teeth and position the head in dorsiflexion by sticking the suture to the acrylic glass with adhesive strips.
10. Translocate the animal on the platform under the operation stereomicroscope. Use 16X magnification.

3. Preparation of the Left Jugular Vein and Injection of FITC-Dextran

Note: For microscopy of the right ear, prepare the left jugular vein.

1. Using a scalpel, create a 5-mm incision in the skin on the left side of the neck in a cranio-caudal direction. Dissect the subcutaneous tissue with a microforceps and microscissors. Either ligate crossing vessels with polyester 8/0 sutures or with electrocoagulation.
2. Free the vein from its adventitia using microforceps and microscissors without touching the vessel.
3. Use the prepared insulin syringe for the injection of the fluorescent dye. Carefully grab the vessel wall with the microforceps, without perforating the vein. Penetrate the distended vessel wall with the syringe and inject FITC-dextran iv.
4. Stop the bleeding after withdrawing the syringe using cotton swabs. Avoid blood and dye contamination of the ear.

4. Positioning of the Right Ear for Intravital Fluorescence Microscopy

1. Transfer the animal on the heating plate to an acrylic glass construction with a slot for the heating plate and a 0.5 cm-high plane for positioning the ear.
2. Fix the animal face down on the heating plate using adhesive strips. Place the relatively strong and convex cartilage at the base of the ear beside the 0.5 cm-high plane for the ear (**Figure 1B**) so that the apical part of the ear can be positioned flat on the plane.
3. Add one drop of room-temperature 0.9% NaCl to the acrylic glass plane in order to position the ear. Place the right ear, with the prearranged sutures on its concave ventral side facing downwards, on the drop of 0.9% NaCl. Using cotton swabs, absorb the drop of NaCl and let capillary forces attach the ear plane to the acrylic glass.
4. Tape the sutures to the acrylic glass to fix the position of the ear.
5. Add one drop of 0.9% room temperature NaCl to the convex dorsal side of the ear. Carefully put one coverslip (0.5 cm diameter) on the ear without compressing the basal vessels entering the ear. Using cotton swabs, remove as much NaCl as possible from under the coverslip in order to minimize the distance between the coverslip and the ear target vessels.

5. Intravital Fluorescence Microscopy and Thrombus Induction of the Right Ear

1. Adjust the intravital fluorescence microscope for FITC-dextran visualization (450 - 490 nm; FT: 510; LP: 520). Use a variable 100-W mercury lamp as a light source. Connect a high-resolution, black-and-white CCD camera to a DVD recorder.
2. Transfer the animal on the acrylic glass containing the heating plate with the fixed abducted ear to the desk of the intravital fluorescence microscope.
3. Using 20X magnification (20X/0.95 numeric aperture) and 20% light intensity, search for a venous vessel 50 - 60 µm in diameter and with an anterograde blood flow of 400 - 600 µm/s.
4. Add one drop of room-temperature water to the coverslip for water immersion of the 63x magnification objective (63X/0.95 numeric aperture). Use a syringe with a 1-mm diameter cannula and place the drop on the objective of the microscope. Add just enough water to contact the coverslip and the objective with the water drop.
5. Immediately after the application of the water drop, begin recording the vessel for 20 s with 20% light intensity for the offline measurement of the diameter and blood flow.
6. Start thrombus induction 5 min after the injection of FITC-dextran. For this purpose, raise the light intensity to 100%.
7. During thrombus induction, close the aperture of the microscope for 2 s within a period of 30-s to check the blood flow. In case of persisting blood flow, open the aperture again. In case of stopped blood flow, observe the vessel for 30 s.

NOTE: The vessel is classified as occluded if the flow stands still for 30 s or more or if the blood flows retrogradely. If the orthograde blood flow starts again, completely open the aperture and continue the thrombus induction until vessel occlusion occurs as described above. During early thrombus induction, ensure that the times when the aperture was closed to check the blood flow are as short as possible in order to maintain almost continuous epi-illumination. Later, during thrombus growth, the vessel is perfused with less fluorescent dye, so it can be observed continuously.

8. Select and occlude 5 vessels per ear. Limit the time of thrombus induction under the microscope to approximately 1 h after the injection of FITC-dextran.

6. Follow-up Activities

1. Perform a wound closure of the neck using transcutaneous polypropylene 6/0 sutures.
2. During recovery from anesthesia, put the mouse back into the cage and warm the animal using infrared light.
3. Transfer the recorded data from the DVD recorder to software allowing the measurement of the diameter of the vessels and velocity of blood flow.

7. Examination of the Left Ear

1. Let the animal recover and eliminate all injected FITC-dextran for 48 h.
2. Rerun the steps described above, this time preparing the right jugular vein and the left ear.

8. Tissue Asservation

1. After intravital fluorescence microscopy of the left ear, sample 0.5 mL of blood from the retrobulbar vein plexus of the eye using a glass capillary. Carefully penetrate the inner palpebral angle with screwing movements, until venous blood flows through the capillary. Collect the probe in an ethylenediaminetetraacetic acid (EDTA) blood tube.
2. After blood sampling, sacrifice the animal by injecting 500 mg/kg bw ketamine into the tail vein.
3. Count the blood cells using a hematology analyzer for a quantitative assessment of leukocytes, erythrocytes, thrombocytes, hemoglobin, and hematocrit.
4. Centrifuge the remaining EDTA blood at 2,500 x g and room temperature for 10 min. Pipette and freeze the blood plasma for further investigations.
5. Using scissors, cut the auricles and fix them in 4% formaldehyde for histological examination.

Representative Results

Effects of Cannabinoid Treatment on Thrombogenesis

Upon injection of 0.05 mL of FITC-dextran, phototoxic thrombus induction leads to an endothelial lesion and the formation of a parietal platelet plug (**Figures 2 and 3**). In the present study, thrombus induction after the ip injection of cannabinoids (5 mg/kg bw) or vehicle resulted in a thrombotic vessel occlusion in all venules (**Figure 4**). In vehicle-treated animals, the time to thrombus formation was 430 s (25th percentile: 330 s; 75th percentile: 637 s). Neither cannabidiol (CBD) nor WIN55,212-2 (WIN) administration showed a relevant influence on vessel occlusion times. However, the endogenous cannabinoid anandamide significantly reduced the time needed for thrombus formation and thrombotic vessel occlusion to 270 s (25th percentile: 240 s; 75th percentile: 360 s, $P < 0.05$ versus vehicle).

To test whether the hydrolysis of anandamide and the subsequent cyclooxygenase-dependent conversion of its product, arachidonic acid, is involved in thrombus formation by anandamide, the unspecific cyclooxygenase inhibitor indomethacin was combined with anandamide in another set of experiments (**Figure 5**). Again, upon injection of 0.1 mL of FITC-dextran, anandamide (10 mg/kg bw) reduced thrombus formation times, as compared to vehicle ($P < 0.05$ versus vehicle). While indomethacin treatment alone had no effect on venular occlusion times, cyclooxygenase inhibition in anandamide-treated animals significantly prolonged thrombus formation to 300 s (25th percentile: 240 s; 75th percentile: 420 s), as compared with the median of 160 s (25th percentile: 100 s; 75th percentile: 200 s) after anandamide treatment only ($P < 0.05$ versus anandamide). Vessel occlusion times after vehicle treatment and indomethacin/anandamide co-administration did not significantly differ².

Figure 1. Preparation of the Jugular Vein (A) and Placement of the Ear for Intravital Microscopy (B). (A) Using the operation stereomicroscope, the right jugular vein is prepared. The sutures for the placement of the left ear are stitched before the injection of FITC-dextran. The wound from the prior dissection of the left jugular vein is closed with transcutaneous sutures (B). The left ear is fixed with polypropylene 7/0 sutures. A coverslip is carefully placed without compressing the basal vessels of the ear. A heating plate maintains the body temperature of the animal during the whole experiment. [Please click here to view a larger version of this figure.](#)

Figure 2. Intravital Microscopy and Thrombus Induction. The same venule is shown before (A) and after (B) thrombus induction at 10X, 20X, and 63X magnification (left to right). The blood plasma is stained with 0.05 mL of fluorescein isothiocyanate-labeled dextran (FITC-dextran, 5%). The thrombus is marked with *. [Please click here to view a larger version of this figure.](#)

Figure 3. Venular Thrombus Induction. The same venule is shown before (A), after 100 s (B), and after 300 s (C) of epi-illumination by means of intravital fluorescence microscopy. Reactive oxygen species are generated by blue light epi-illumination (450 - 490 nm) of FITC-dextran and impair the endothelium. Thus, the platelets are activated and adhere to exposed subendothelial structures, resulting in primary parietal thrombus formation (B) and, subsequently, in complete thrombotic vessel occlusion (C). This figure has been modified from Grambow³. The thrombus is marked with *. [Please click here to view a larger version of this figure.](#)

Figure 4. Flow Chart Displaying the Experimental Protocol. Cannabinoid treatment by ip injection of the respective cannabinoid (5/10 mg/kg bw) was performed 30 min before IVM and the induction of thrombus formation in the right ear on day 0. IVM was limited to 1 h. 47 h later, on day 2, the same protocol was applied to the left ear of the mouse before sampling the blood. This figure has been modified from Grambow³. Please click here to view a larger version of this figure.

Figure 5. Venular Thrombus Formation after Single Cannabinoid Treatment and Cyclooxygenase Inhibition. Occlusion times of venules in mice undergoing light/dye thrombus induction. **(A)** Animals were treated with the DMSO-containing vehicle (VEH) or with the cannabinoids anandamide (AEA), WIN55,212-2 (WIN), or cannabidiol (CBD) (5 mg/kg bw; n = 5). 0.05 mL of 5% FITC-dextran was injected iv prior to thrombus induction. In another setting, 0.1 mL of 5% FITC-dextran **(B)** anandamide (10 mg/kg bw) was combined with indomethacin (Indo) (5 mg/kg bw) to assess the impact of cyclooxygenase-dependent products on thrombus formation by anandamide (n = 5). Kruskal-Wallis one-way ANOVA on ranks was followed by Dunn's post hoc analysis **(A and B)**. The values are given as the median and IQR (5th, 25th, 75th, and 95th percentiles). * P < 0.05 versus vehicle, # P < 0.05 versus anandamide, § P < 0.05 versus indomethacin. This figure has been modified from Grambow³. Please click here to view a larger version of this figure.

Discussion

There are several critical steps for the successful thrombus induction in the earlobe of SKH1-Hr^{hr} mice. For troubleshooting, the respective steps of the protocol are indicated in parenthesis.

Examination conditions are ideal in young animals at the age of 4 - 6 weeks and with low cornification of the epidermis. In older animals, the quality of visualization of the vessels is worse and less comparable due to the higher distance between the skin surface and the target vessels (step 1.1).

To prevent the extravasation of FITC-dextran in the area of examination, the fixing sutures must be placed as marginally as possible. In proximity to the stitches, fluorescent dye can extravasate and reduce the contrast between the extravascular space and the vessels. This extravasation

of the dye progresses slowly. If the sutures are stitched as mentioned above 15 min prior to the injection of FITC-dextran, good examination conditions of intravital microscopy are ensured (step 2.8).

FITC-dextran is slowly eliminated renally. The combination of the fluorescent dye with high molecular dextran (150 kDa) delays extravasation and excretion. In the present study, the time for microscopy and thrombus induction was limited to 1 h to prevent the influence of excretion and low fluorescent dye plasma concentrations on thrombus formation time.

While filling the syringes, no air bubbles should remain for the injection, because even small iv-administered air bubbles in the syringe can be lethal for the animal (step 2.5). When pulling the syringe out of the jugular vein after the iv injection, regular bleeding occurs (step 2.6). Contamination of the subsequently examined ear with blood or FITC-dextran makes intravital microscopy substantially difficult or even impossible. Thus, preparation of the contralateral ear and jugular vein is recommended.

The injection of the fluorescent dye must be as precise and complete as possible, because incorrect dye administration would markedly influence the occlusion times (step 3.3). Injections into the tail vein and retrobulbar vein plexus are not recommended. They are not as reliable as administration into the jugular vein, even though they are less invasive for the animal. Therefore, FITC-dextran was injected into the mouse jugular vein to assure the immediate and complete uptake of the dye. If continuous access to the carotid artery or jugular vein is necessary in the experimental protocol, surgical implantation of a subcutaneously tunneled catheter can be performed.

The coverslip must be carefully applied, without any additional pressure (step 4.5). Otherwise, the blood flow in the whole ear is slowed due to the compression of the basal vessels, which could decrease the occlusion times during thrombus induction. The accurate placement of the coverslip can be verified with the stereomicroscope. The venules have to be filled continually, especially at the edges of the coverslip. The coverslip must be used to guarantee the contact of the water drop with the objective of the intravital fluorescence microscope. The immersion has to be obtained during the entire thrombus induction in order to assure the epi-illumination of the vessel with 100% light intensity. The best way to achieve the stable placement of the water drop is by using the coverslip. Putting the drop on moisturized translucent plastic wrap or directly onto the skin causes drain of the water and inconstant immersion.

Significance and Limitations of the Earlobe of the Hairless SKH1-Hr^{hr} Mouse

The SKH1-Hr^{hr} hairless mouse allows for direct functional imaging of the vessels in the earlobe using intravital microscopy^{2,4,5}. The histology of the ear resembles the anatomy of human skin². The entire microvascular network, consisting of venules, arterioles, and capillaries up to 100 µm in diameter, can be visualized and examined in real time. This makes the ear of hairless mice a suitable model for the study of wound healing^{6,7}, axial-pattern flaps^{2,5}, macromolecular leakage⁵, and microvascular thrombus formation^{8,9,10}. The availability of vessels up to 100 µm in diameter is a limit to the model. Shear stress, blood flow, and vessel architecture differ in small and large vessels. Therefore, models like the carotid artery or the femoral vessels may be more suitable for studies focusing on macrovascular thrombus formation.

All alternative models of intravital visualization of microcirculation, such as the cheek of the hamster¹¹, the dorsal skinfold chamber of the mouse^{12,13}, or the cremaster muscle of the rat^{9,10} require surgical preparation. The vessels of the ear of the hairless mouse are accessible without any risk of tissue damage by surgery, so there is no influence on the measurement parameters by inflammation, vasoconstriction, and activation of hemostasis in the earlobe of the hairless mouse¹⁴. Even though no surgical preparation is necessary, the image resolution and clarity are comparable to other models (e.g., the dorsal skinfold chamber and cremaster preparation). In order to achieve a high imaging quality, the protocol must be followed thoroughly, and young mice with less cornification of the dermis have to be used.

Due to their superficial localization, the vessels of the ear can easily be studied by intravital fluorescence microscopy. They enable thermoregulation in the animal through their adjustment of the vessel diameter. Therefore, both room and body temperature have to be standardized to achieve reproducible results. All earlobe vessels represent peripheral vessels in dermal tissue. Compared to central vessels, peripheral vessels are characterized by different histological structures and receptor expression. Therefore, other models (e.g., the preparation of mesenteric venules and arterioles) may be more reasonable for the examination of specific issues concerning central vessels.

Another limitation of the described model is the restriction associated with using hairless SKH1-Hr^{hr} mice, which are not as common as other mouse strains. Therefore, breeding transgenic hairless mice can be laborious and expensive. Chemical and mechanical hair removal can cause local inflammation and does not remove the hair roots, which may impair visualization quality. As good visualization quality and low distance between the surface and the target vessel is crucial for reliable thrombus induction; other models (e.g., the dorsal skinfold chamber) of the mouse may be more suitable for studies that require certain mouse strains with fur. On the other hand, the earlobe model enables the simulation of various pathological conditions. For example, the microcirculation in critically perfused tissue can be examined after the ligation of two of the three neurovascular bundles¹⁵. Analysis of microcirculation during wound healing is another suitable example for the use of the ear of the hairless mouse model¹⁶.

For some experimental questions, it is important to examine the same animal at different time points to assess the time sequence of a treatment. In the recently published experimental study, thrombus induction in the left ear was not altered by prior treatment of the right ear³. Therefore, another advantage of the model is the possibility of thrombus induction in each ear of the same mouse at two different time points. Concerning animal protection, the experimental procedure is minimally invasive for the animals, and the mice do not have to recover from surgery or carry a dorsal skinfold chamber between the experiments. In every animal, at least five appropriate vessels per ear can be occluded by phototoxic thrombus induction, so much data can be collected with a small number of animals.

Significance and Limitations of Intravital Microscopy and Thrombus Induction

Intravital fluorescence microscopy allows the visualization of microcirculation in real time⁵. After iv administration, FITC-dextran stains the blood plasma. It enables the observation of thrombus growth from the beginning of the induction until complete vessel occlusion. White and red blood cells can be identified as gaps in the contrast medium. For further investigations (e.g., granulocyte-endothelium interactions), white blood cells can be stained with rhodamin-6G.

The recording and offline analysis of the experiment enables the *in vivo* measurement of red blood cell velocity, arteriolar vasomotion, capillary density, and microvascular diameter. These parameters play a significant role in thrombus formation, flap perfusion, and wound healing. Observation by intravital microscopy can directly and continuously quantify these dynamic perfusion parameters and their alteration during the experiment⁵. Other techniques, like xenon washout, tissue oxygen levels, laser Doppler, or dye diffusion, are also minimally invasive, but they are restricted by the indirect measurement of blood flow. This may affect the validity of the experimental results. Therefore, direct methods are preferred.

The reaction of the fluorescent dye and the light of a certain wavelength results in the release of reactive oxygen species, which locally damage the endothelium¹⁷. The exposition time of the fluent blood particles is 1,000x less when compared to the endothelium. Therefore, the thrombogenic effect is primary due to a phototoxic endothelial lesion and not due to direct phototoxic platelet activation¹⁸. Platelets are activated through contact to the exposed subendothelial matrix and form a platelet plug¹⁹ (**Figure 3**). This mechanism of thrombogenesis plays an outstanding role in many situations, such as unstable angina pectoris and vascular anastomosis.

Light/dye thrombus induction is less invasive than the alternative methods of creating endothelial lesions through balloon catheter²⁰, electric current²¹, laser²², or inflammation¹⁹. Thrombus induction with light/dye also acts strictly locally in the light beam of the objective. Therefore, neighboring vessels are not directly affected and can be used for subsequent thrombus induction. Light/dye thrombus induction can be performed in both venules and arterioles. In the present study, venules were exclusively treated because epi-illumination of arterioles can cause vasospasm, which might affect the occlusion times¹⁹.

Anesthesia was performed using the combination of xylazine and ketamine, which is established in veterinary and experimental medicine. The drugs were injected ip. With the abovementioned dosage, sufficient anesthesia with surgery tolerance for 30 min and sleep for 1.5 - 2 h was achieved.

The ear of the hairless SKH1-Hr^{hr} mouse is well established in wound healing and flap research. Several studies have used the model successfully for thrombus induction and thrombolysis^{3,23,24,25,26}. If the protocol is performed properly, intravital microscopy in the earlobe of the hairless SKH1-Hr^{hr} mouse is a reliable, easy, and efficient tool for the study of microcirculation and thrombus formation. It is simple to simulate various pathological conditions, while the model offers an excellent experimental setting to assess crucial parameters of microcirculation *in vivo*.

Disclosures

The authors have nothing to disclose.

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