

FLUORESCENCE MICROLYMPHOGRAPHY: DIAGNOSTIC POTENTIAL IN LYMPHEDEMA AND BASIS FOR THE MEASUREMENT OF LYMPHATIC PRESSURE AND FLOW VELOCITY

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ABSTRACT

Fluorescence microlymphography (FML) is an almost atraumatic technique used to visualize the superficial skin network of initial lymphatics through the intact skin of man. Visualization was performed with an incident light fluorescence microscope following subepidermal injection of minute amounts of FITC-dextran 150,000 using microneedles. Emanating from the bright dye depot, the surrounding network of microvessels is filled, documentation performed by photography or video film. In congenital Milroy lymphedema, a lack of microlymphatics (aplasia) is typical while in other primary lymphedemas and in secondary lymphedema after mastectomy or irradiation of proximal lymph nodes, the network remains intact but the depicted area is enlarged. Lymphatic microangiopathy characterized by obliterations of capillary meshes or mesh segments develops in phleboedema with trophic skin changes, progressive systemic sclerosis and Fabry's disease. In lipedema, lymphatic microaneurysms are stained. Microlymphatic pressure may also be measured using FML. For this purpose, glass micropipettes are inserted into the capillaries by means of a micromanipulator and pressure is determined by the servo-nulling technique. Normal subjects produced significantly lower pressure (7.9 ± 3.4 mmHg) compared to patients with primary lymphedema (15.0 ± 5.1 mmHg, $p < 0.001$). This characteristic

lymphatic hypertension may be improved by complex physiotherapy or local application of prostaglandins. Additionally, a modification of the FML procedure can be used to measure lymphatic capillary flow velocity in controls and patients.

FML is suited to confirm the clinical diagnosis of lymphedema, contributes to distinguish among various forms of edema, and is useful in clinical research. In addition, FML has also become a tool for experimental animal studies including the depiction of gastric microlymphatics, the measurement of flow velocity in the naked mouse tail, and in evaluation of lymphangiogenesis in a model of Milroy disease.

Keywords: fluorescence microlymphography, lymphedema, Milroy disease, lymphatics, lymphatic pressure, lymphatic flow velocity, lymphangiogenesis, microangiopathy

Large molecules in the tissues are primarily drained by the lymphatic system. With the aim to render the initial lymphatics visible in vivo and in clinical medicine, the idea arose about 1980 to inject minute amounts of fluorescent macromolecules into the interstitial space of toes and distal leg skin. The commercially available fluorescent dextrans of different molecular sizes had previously been given intravenously to experimental animals in order to detect blood capillary leakage (1). After preliminary tests in the big toe of the first author and then in

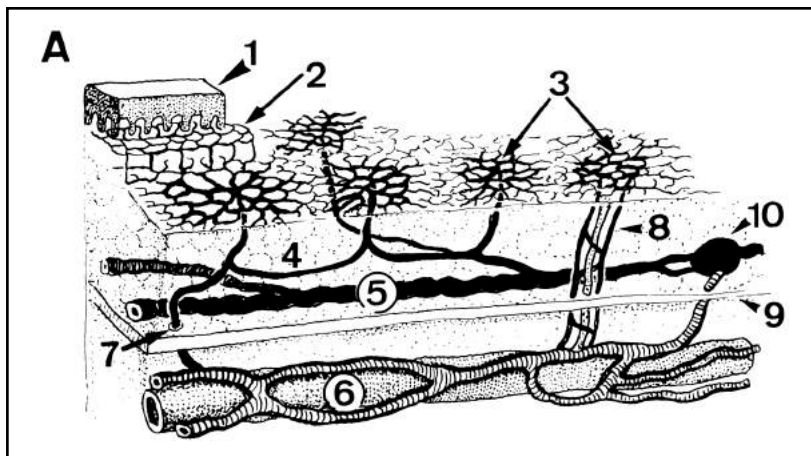


Fig. 1: Anatomy of the microlymphatics in human skin according to Kubik. Two networks of initial lymphatics are superposed (2,3) and connected by channels running perpendicular to the skin surface (4). There are also connecting vessels (7,8) to deeper epi- and subfascial lymphatics (5,6). The fascia is indicated by 9. FML depicts the superficial network of the epidermis (1). Modified from Kubik (3).

other healthy volunteers, it became evident that subepidermal injections of 0.01-0.05ml FITC-labeled dextran with a molecular weight of 150,000 were well tolerated and offered a way to visualize the lymphatic capillary network of the skin (2).

The technique was called fluorescence microlymphography (FML). First, it was used in healthy controls and patients with primary lymphedema beginning after puberty (2). Approximately during the same period, Kubik (3) succeeded in staining the superficial and deeper network of lymphatic skin capillaries and the precollectors by injecting Indian ink into the skin of human cadavers. The detailed anatomy analyzed by him is illustrated in *Fig. 1*. The microlymphatic plexus in the leg of a human fetus had previously been described by Kampmeier in 1928 (4).

To date, the characteristic network of human skin microlymphatics, which is denser in the lower leg than in the forearm (5), has been studied in healthy controls, different forms of lymphedema and other disease entities (5-21). Moreover, the technique has served as a basis for measurements of microlymphatic pressure (22-26) and flow velocity (27,28) in control subjects and patients.

This article reviews the diagnostic potential of the FML method in clinical medicine and summarizes the findings obtained in experimental research. It should be emphasized that the technique was first used in human digits and limbs and only later transferred to animals (29-33).

TECHNIQUES

Conventional microscopes with incident light illumination and fluorescence filters allow visualization of the superficial skin microlymphatics through the intact skin of man. For documentation of dynamic phenomena and electronic processing of images, we used video microscopy systems (2,8,11), which were mounted on the flexible arm of a heavy support. In this way, adaptation of the instruments to different parts of the body was facilitated. On the other hand, morphological studies are also possible by using simple on-light fluorescence microscopes and camera.

FITC-dextran 150,000 is available as a powder (Sigma). Before use, a 25% solution is dissolved in physiologic saline solution and sterilized. Minute amounts (0.01-0.05 ml) of

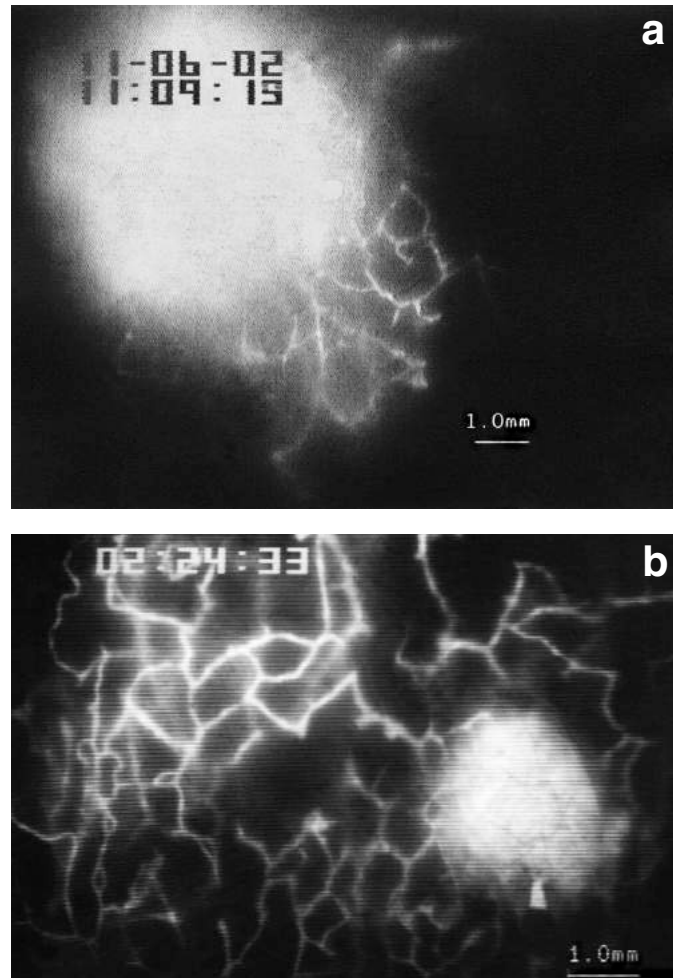


Fig. 2: a) FML at the medial ankle in a normal control. The large and bright fluorescent spot represents the dye depot located in the epidermis. Only few meshes of the superficial network are visualized. b) In a patient with sporadic primary lymphedema with onset after puberty, a much larger network is depicted from the original dye depot.

fluorescent dye are injected into the subepidermal layer (2,5-21) by means of microsyringes and steel microneedles with an outer diameter of 0.2 mm (Hamilton®). The injection site is selected according to the region of interest. All accessible parts of skin and mucosa are suitable for examination. In most studies the patients rested comfortably in supine position. For investigating the effect of gravity, the patient may also be tilted (24). Immediately after injection, the bright dye depot is evident using the microscope. Originating from the fluorescent spot, the

adjacent network of initial lymph vessels is filled and becomes easily visible (*Fig. 2a,b*). If the depot is not precisely located in the subepidermal layer, depiction may fail. Images are recorded by photography or video.

The technique using thin needles and minute amounts of fluorescent dye is almost non-invasive and with some training, it becomes easy to perform. It has even been used in children with Milroy disease (7,11). Among several thousand examinations, side effects occurred only in a few patients and consisted of transient reddening, swelling,

and itching at the site of microinjection. In one instance, however, generalized urticaria required intravenous administration of steroids. We advise having these medications available on site.

For measuring microlymphatic pressure, the initial lymphatics are visualized by FML and then punctured by glass micropipettes with a diameter of 7-9 μ m mounted on a micromanipulator. This difficult technique requires skill on the part of the investigator. A servo-nulling pressure system, designed by Intaglietta and coworkers (34) and manufactured by IPM San Diego®, and a suited recorder are connected. Details have been published previously (22-26).

Measurements of flow velocity in lymphatic capillaries do not require additional equipment. However, a special procedure is utilized as performed during replay of video films described in two previous reports (27,28).

Diagnostic Tool in Different Forms of Lymphedema

FML may be used to confirm the clinical diagnosis of lymphedema. In normal skin, only a few meshes of the superficial microlymphatic network are stained (2,5-21, *Fig. 2a*) and the dye is quickly drained into the deeper invisible channels. In primary lymphedema of the leg with onset after puberty (2,8,10-12,19,23,25), however, and also in secondary arm lymphedema after mastectomy (17), the extension of the depicted network is enhanced (*Fig. 2b*). At the medial ankle, the maximal distance between the outer border of the dye depot and the most distant visible lymph vessels normally does not exceed 12 mm (8,11). An extension higher than 12 mm strongly supports the presence of lymphedema. Because of insufficient drainage of the dye into the hypoplastic or proximally blocked deep lymph collectors, FITC-dextran 150,000 expands more into the intact superficial network than in healthy subjects (8,11).

In some patients, the phenomenon of cutaneous reflux, well known from conventional lymphography with contrast media, is observed at the microvascular level (11,28). The macromolecular dye moves into deeper invisible channels and reemerges in network segments away from the part directly filled from the dye depot. The phenomenon is due to a reflux of the stained fluid through communicating vessels without functioning valves. It occurs in different forms of lymphedema and phleboedema but not in healthy controls. As has been shown in some lymphedema patients (28), one with a non-healing traumatic ulcer, lymphatic reflux from deeper vessels may be rhythmic (see later in the section on flow velocity).

In both primary and postmastectomy lymphedema, the morphology and the diameter of the superficial skin microlymphatics are normal (2,10-12,17,19) and these two parameters do not aid in differential diagnosis. Extension of the depicted network is significantly increased in both forms and does not distinguish either form.

On the other hand, FML is an excellent way to distinguish between Nonne-Milroy disease and other, more common forms of lymphedema. Milroy hereditary lymphedema present at birth is characterized by aplasia of the initial lymphatics (7,10,11). Several depots of FITC-dextran 150,000 made by a skilled technician failed to stain any microvessel in the edematous parts of the limbs. In a Milroy family with typical genealogical tree, four members belonging to two generations exhibited lack of skin microvessels in their swollen ankles (7). Outside of the edematous limb segments, however, the networks were normally developed and patterned. In an adolescent brother and his sister in another Milroy family, lymphedema was unilateral. Aplasia in them was confined to the diseased right leg (*Fig. 3*) (11). Absence of initial lymphatics was also found in a mouse model of Milroy disease, in which inactivation of VEGF-C and -D leads to physical signs similar to human lymphedema (32). Rare

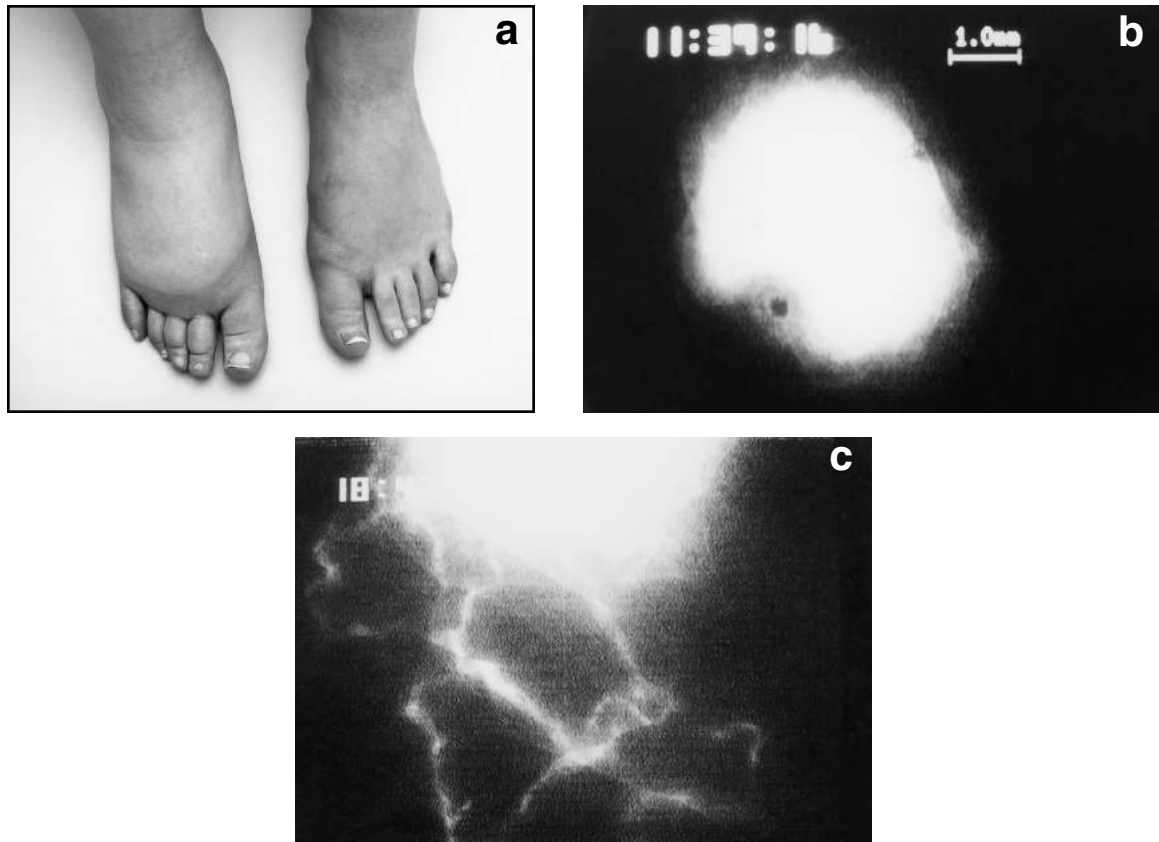


Fig. 3: a) 18 year old young lady with Milroy disease limited to the right leg and foot. b) At the right foot dorsum with manifest edema, it is not possible to visualize any lymphatic capillary by FML (aplasia). c) On the healthy left side a few normal meshes appear. Modified from Bollinger and Fagrell (11).

exceptions to the rule occur. In four patients, the initial lymphatics were present but markedly enlarged (10,11), a finding unknown in other forms of lymphedema. Patients with sporadic primary lymphedema have normal capillary diameters at the medial ankle (average $56.3 \pm 9.0\mu\text{m}$, range 45 to $73\mu\text{m}$) (10). The diameter values in the exceptional Milroy patients just mentioned all exceeded $90\mu\text{m}$ (range 92 to $112\mu\text{m}$) (10).

In patients with primary lymphedema and late manifestation, repeated erysipelata may induce morphologic changes in skin microlymphatics. In these cases, multiple obliterations interrupt the meshes of the capillary network (11). Most likely the damage

to the initial lymphatics caused by infections is the reason that primary non-hereditary lymphedema may deteriorate after recurrent erysipelata.

Diagnostic Contributions in Other Forms of Edema

Morphological changes in the superficial skin microlymphatics are also found in other diseases involving the extremities. In advanced phleboedema, especially at sites with trophic skin lesions and recurrent leg ulcers, the blood capillaries are dramatically altered (11). Enlarged and tortuous capillary convolutions resembling glomeruli may be

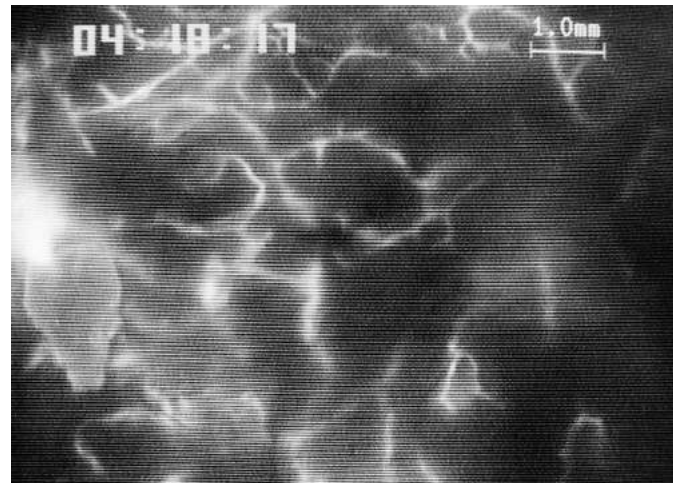


Fig. 4: In a patient with severe chronic venous insufficiency and indurated skin edema, FML at the lower leg shows a large network of lymphatic capillaries. The meshes are no longer regularly shaped, but interrupted in part by obliterations (lymphatic microangiopathy).

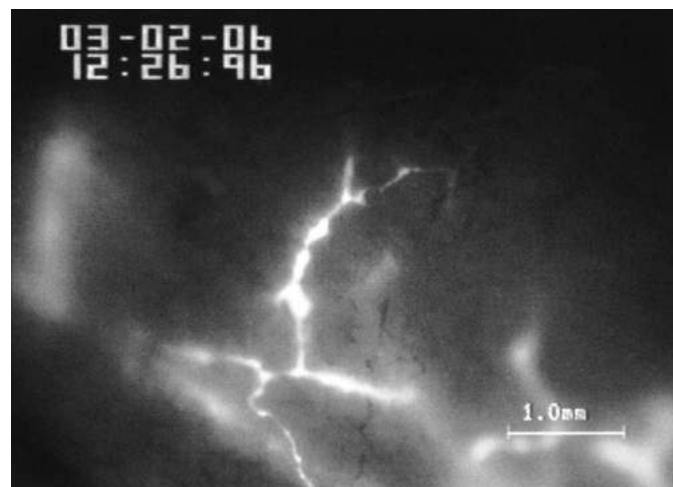


Fig. 5: FML in a female patient with lipedema. In the center, microlymphatic vessel shows the typical variations of caliber with enlargements (microaneurysms) followed by narrow segments. Reproduced with permission from Amann-Vesti et al (18).

obliterated by microvascular thrombosis. Rarefaction of blood microvessels and localized skin ischemia are a consequence (11). But the microlymphatics are also affected in severe phleboedema. Lymphatic microangiopathy becomes a prominent feature (6,8,11). Single meshes are interrupted (Fig. 4) or whole network areas obliterated,

and they are no longer filled by the macromolecular dye. The obstructions of microlymphatics may contribute to edema formation in the lower legs of patients with severe chronic venous insufficiency. The indurated swelling is not due to venous stasis alone, but aggravated by deficient lymphatic drainage.

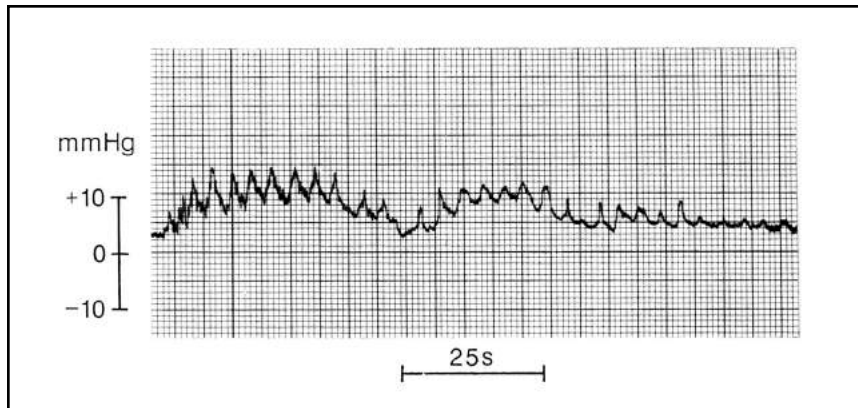


Fig. 6: Original recording of skin lymphatic capillary pressure. The small peaks displayed correspond to fluctuations induced by respiration, and frequency fluctuations are also seen.

Another form of lymphatic microangiopathy is observed in patients with lipedema (18). In the limb segments affected by the swelling, the lymphatic microvessels exhibit marked variations of caliber. Some capillary segments are enlarged and appear aneurysmatic; others are of normal width or even narrowed (Fig. 5). So far, this combination has not been found in any other disease. FML is a useful tool for diagnosis of this still poorly understood condition (18).

Fabry's disease may become complicated by leg lymphedema characterized by marked fragmentation of the microlymphatic network (20). The changes observed are not discernible from other forms of lymphatic microangiopathy.

Microlymphatic alterations have also been documented in patients with myxedema (13) and psoriasis (15). In progressive systemic sclerosis, pronounced changes of blood capillaries at the nailfold are of considerable diagnostic value (11). The lymphatic microvessels are interrupted, rarefied or lacking in part (16). In split-skin grafts, fragments of the initial lymphatics are mostly filled by cutaneous reflux (21). In finger and upper limb reimplantations, newly formed microlymphatics often bridge the scars if enough time has elapsed after the operation (14).

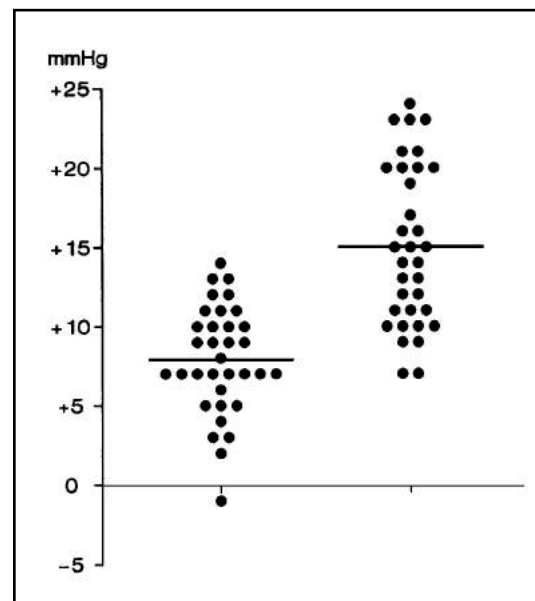


Fig. 7: Microlymphatic hypertension at the distal forefoot in patients with primary leg lymphedema and late manifestation. There is some overlapping with the values measured in the healthy controls and mean values are indicated by bars for both groups. Reproduced with permission from Zaugg-Vesti et al (23).

Lymphatic Capillary Pressure and Flow Velocity

Normal microlymphatic pressures in the lower leg and foot dorsum fluctuate with

respiration (22,23). In addition, slow frequency fluctuations are found (*Fig. 6*). In healthy volunteers, changes from supine to sitting position induce a significantly larger increase in venous blood pressure (from 6.8 ± 3.4 to 53.3 ± 4.1 mmHg) than in lymphatic pressure (from 3.9 ± 4.2 to 9.9 ± 3.0 mmHg) at the dorsum of the foot (24). This interesting observation is probably due to enhanced contractile activity of lymphatic collectors and precollectors during orthostasis while the veins are not able to pump actively at all.

Patients with primary lymphedema exhibit microlymphatic hypertension (*Fig. 7*) (23). In a control group of normals ($n=34$), the pressure averaged 7.9 ± 3.4 mmHg (range -1 to +14 mmHg) while patients with lymphedema ($n=34$) averaged 15.0 ± 5.1 mmHg (range 7 to 24 mmHg). The difference was statistically significant ($p<0.001$). Moreover, the prevalence of pressure fluctuations with low frequency is increased in the patients compared to controls. Without referencing the previous work of the Zurich group (22-25), Allegra and coworkers (19) repeated the measurements of microlymphatic pressure in patients with lymphedema and confirmed the presence of microlymphatic hypertension in this condition.

The treatment of lymphedema patients by combined physical therapy according to Foeldi, Foeldi and Kubik (35) not only results in a marked reduction of edema but also improves microlymphatic hypertension significantly ($p = 0.01$). In our own study (25), the mean pressure of the initial lymphatics dropped from 12.8 ± 5.7 mmHg (range 6-27 mmHg) to 5.9 ± 4.5 mmHg (range 1-11 mmHg) and with continued compression therapy, the reduction of lymphatic hypertension could be maintained over weeks.

In addition, microlymphatic pressure measurements provide a way to evaluate the effect of drugs on microvascular dynamics. By applying isoprostane 8-epi-prostaglandin F2 alpha by percutaneous route, the high intravascular pressure in patients with

primary lymphedema was decreased (26). The mean values dropped from 19.8 ± 12.1 to 10.0 ± 7.7 mmHg ($p<0.05$) whereas in the cohort of normal controls, no significant effect was observed after drug application. The decrease in hypertension in the patients may be due to enhanced contractility of still intact lymphatic collectors draining the fluid from the initial lymphatics.

By modifying the technique of FML, it also is possible to measure flow velocity in the microlymphatic network of healthy subjects (27) and lymphedema patients (28). In normals, the filling velocity of the microvessels with FITC-dextran 150,000 from the intradermal dye depot was relatively high (median velocity 0.510 mm/s); the velocity under resting conditions, however, was quite low (median value $9.7 \mu\text{m/s}$). Patients with primary lymphedema had significantly ($p<0.05$) higher filling velocities in single vessels of the foot dorsum (0.89 ± 0.43 mm/s) than normal controls (0.55 ± 0.39 mm/s), whereas resting velocities were almost identical in both groups studied (28).

In 4 of 16 patients examined, rhythmic reflux of the fluorescent contrast medium was observed at a distance from the network depicted directly from the dye depot (28) (*Fig. 8*). The mean frequency of the pulsatile cutaneous lymphatic reflux recorded by video densitometry averaged 51 ± 53 sec. These findings suggest that the cutaneous backflow originates from deep lymphatic segments with rhythmical contractions. Pumping collector or precollector segments induce rhythmic flow peaks that are transmitted through insufficient connecting channels to the skin surface (28).

Experimental Animal Research

Soon after the establishment of the method in clinical medicine and research (2,6-8), FML also became a tool in experimental medicine. Nagata and Guth injected FITC-labeled bovine serum albumin into the mucosa of the rat stomach and visualized the

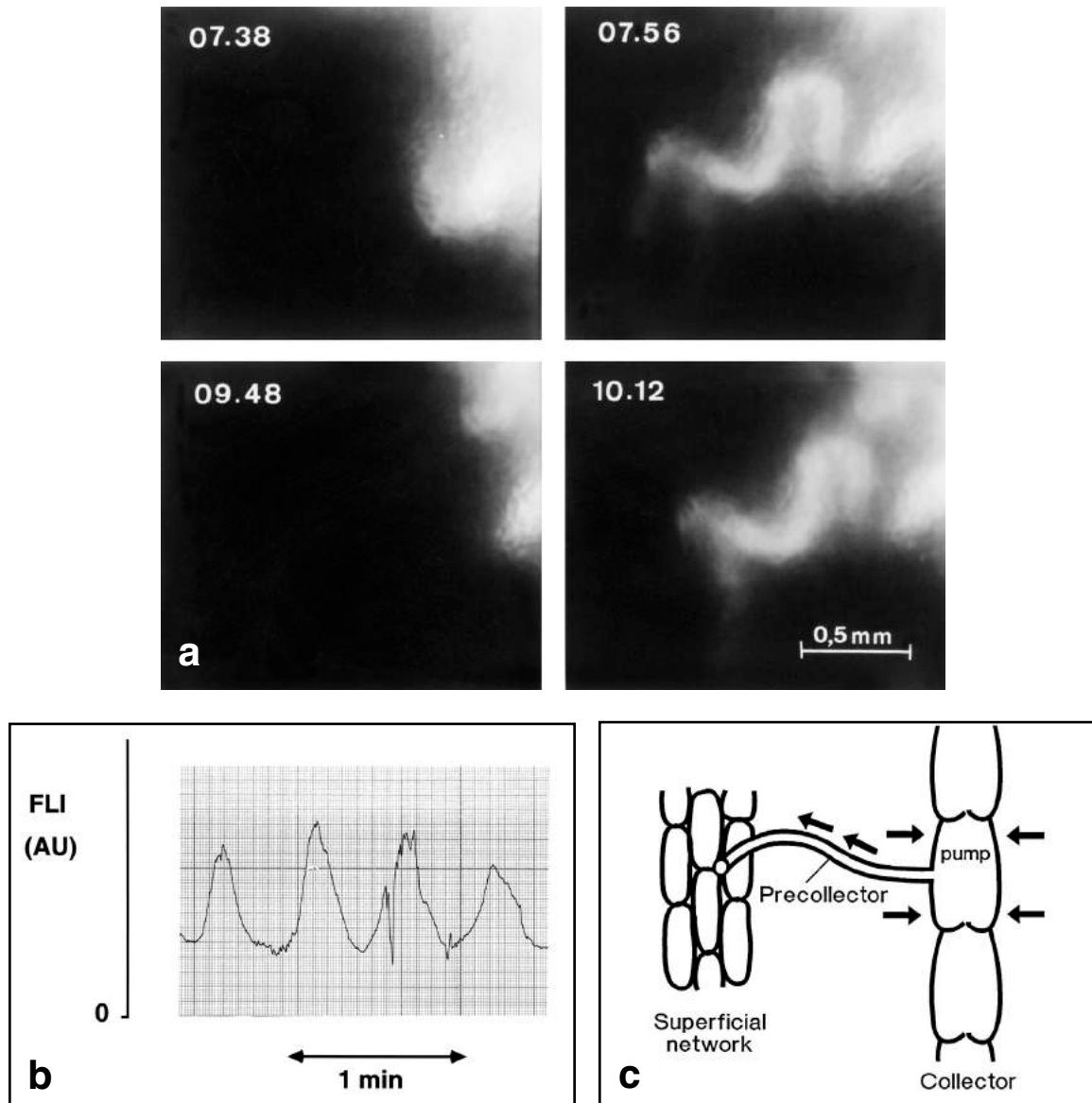


Fig. 8: a) FML at the foot dorsum of a patient with primary lymphedema and late onset. Times (in minutes) after FITC-dextran injection are indicated for the 4 pictures in the upper left. At 7.56 minutes, an enlarged and tortuous lymphatic microvessel is filled from deeper channels. The same vessel appears again at 10.12 minutes. b) Periodic cutaneous reflux may be documented by videodensitometry. The peaks correspond to the superficial arrival of the fluorescent macromolecules. Fluorescence light intensity (FLI) is given in arbitrary units (AU). c) Concept of rhythmic cutaneous reflux in patients with primary lymphedema (pumping of lymph from deep collector or precollector segments through insufficient communicating vessels to the superficial network). Figure 8a reproduced with permission from Fischer et al (28).

anatomy of the gastric microlymphatics in vivo (29). Leu and coworkers in Boston used FML with FITC-dextran in order to depict

the microvessels of the naked mouse tail and to describe their morphology (30). In addition, they succeeded in measuring flow

velocity by a novel photobleaching procedure (30). In another paper, the authors found no functional lymphatics within a murine sarcoma (31). As already mentioned, Makinen and coworkers confirmed the clinical finding of microlymphatic aplasia (7,11) in a mouse model of Milroy disease (32). Also by means of FML, Boardman and Swartz explored how lymphangiogenesis is guided by interstitial flow (33).

CONCLUSION

The technique of FML not only allows the visualization of the initial lymphatics of the skin, but has provided the basis for studying physiology and pathophysiology of the lymphatic system in vivo in humans. Furthermore, the technique has subsequently been applied in animal models to study lymphangiogenesis in different settings and growth factors have been identified that either stimulate (e.g., in lymphedema) or reduce lymphangiogenesis. Based on this knowledge, a new field has been developed for the use of FML. New therapeutic options will be available and FML might be a tool to visualize the effect of blocking or stimulating growth factors for lymphatic vessels in humans in vivo.

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