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Review

Measuring gradients in body fluids – A tool for elucidating physiological processes, diagnosis and treatment of disease

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1. Introduction

There is a growing realization that the tissue microenvironment, consisting of the extracellular matrix (ECM) and the interstitial fluid (IF), has a central role in the development of various diseases, including cancer. Bussard and Smith demonstrated how human breast cancer cells could be re-directed to normal breast epithelial state by placing them in a normal mammary gland microenvironment, suggesting dominance of the tissue microenvironment over cancer cell fate [1–4]. *Vice versa*, the tumor interstitial fluid (TIF) has been given enhanced focus in recent years, especially as a source for biomarkers of disease [5,6]. This is based on the assumption that disease-induced perturbations result in an altered relationship between the tissue specific proteins shed into the microenvironment. These extracellular proteins are transported into the systemic circulation, making them available for sampling in the blood, although heavily diluted [7]. IF is also a valuable sample to study tissue fluid balance and pathological oedema. IF can be used to determine colloid osmotic pressure and tissue hydration, as well as reveal production of biologically important substances for paracrine signaling in the tissue [8–10].

In parallel with this realization, a rapid advancement in mass spectrometry (MS) has provided new tools to dig deeper into the human proteome, and both blood and tissue-based proteomes have been extensively studied. Although the focus has moved from the blood to IF, the majority of published studies on IF proteomics provide qualitative assessments of selected disease proteomes [11–16], semi-quantitative comparison to other tissue proteomes through *e.g.* spectral counting [17], or relative quantification methods such as iTRAQ [18,19]. Absolute quantification with selected-reaction monitoring is an important method for validation of suggested biomarkers in blood [20], but are not as frequently employed on IF samples. The outcome of all these analytical methods are strongly dependent on the choice of starting sample material. Despite the overwhelming number of studies focusing on biomarker discovery, no successful clinical biomarkers have yet been detected through such studies [21,22]. The development and qualification pipeline following discovery of a new biomarker candidate is time-consuming and costly, further complicated due to many biomarker studies with inappropriate design and poorly defined sample starting points [23].

One important, often disregarded, aspect in biomarker discovery is the protein load transported into blood from the tissues in the body not affected by disease, which is not sampled in most biomarker studies. Uhlén et al. used quantitative transcriptomics and micro-array based immunohistochemistry to analyze the tissue specificity and protein expression for > 90% of the putative protein-coding genes in the human genome, and found that almost half of these were expressed in all analyzed tissues [24], with a minor subset of proteins being expressed in a tissue-enriched manner. This makes the *quantification* of interesting proteins and other substances in the extracellular environment even more crucial, to determine origin as well as local and systemic relevance.

It is necessary to move beyond a qualitative approach, because it is not the mere presence of a substance that makes it a disease biomarker or a central actor in disease development and pathophysiology, but rather the quantity of such substances in tissue. The gradient of the substance between the interstitium and systemic circulation can be a useful tool to interpret the origin of such compounds, as exemplified for CA-125, VEGF and osteopontin by Haslene-Hox et al. [2]. To be able to acquire such quantitative and physiologically relevant information, there are several aspects to experimental design that need to be met.

This review will give an overview of the major fluid compartments in the body, summarize methods for isolating tissue IF needed to calculate the tissue-to-blood gradient, and discuss how such gradients can be affected by properties of the tissue interstitium and circulation. A system-level understanding of body fluids and a truly quantitative approach is necessary to fully encompass pathophysiology of a disease and the interplay of various disease associated molecules. To obtain such an understanding, relevant samples must be analyzed in a quantitative way with valid controls.

Abbreviations: IF, interstitial fluid; ECM, extracellular matrix; TIF, tumor interstitial fluid; MS, mass spectrometry; CSF, cerebrospinal fluid

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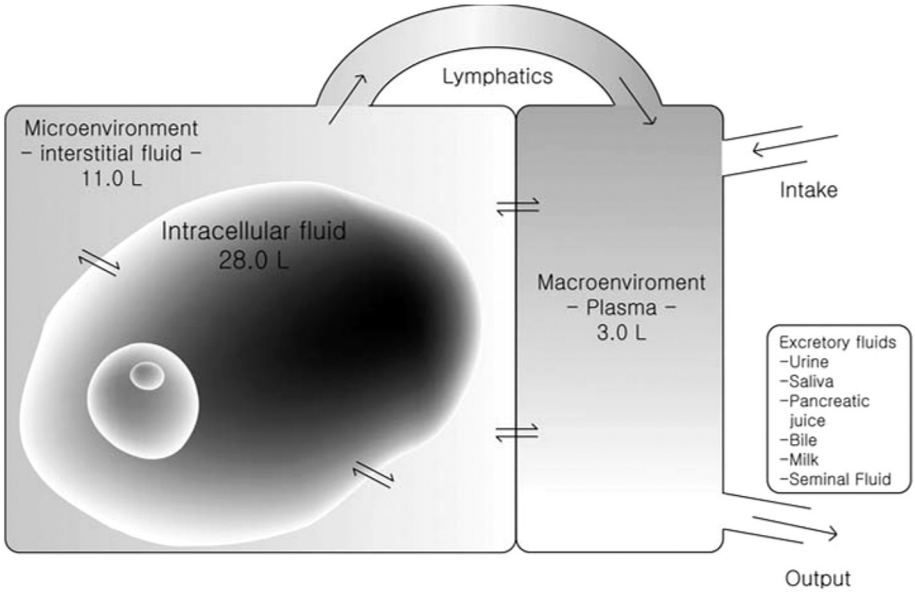

Fig. 1. Overview of body fluids and the internal environment. In an average 70-kg person, the total body water is about 60% of the body weight (approx. 42.0 L). The intracellular fluid constitutes about two-thirds of the total body water (approx. 28.0 L), whereas the extracellular fluid constitutes the rest (approx. 14.0 L). The interstitial fluid comprises > 75% of the extracellular fluid (approx. 11.0 L), whereas plasma comprises the remaining 25% (approx. 3.0 L). From [87].

2. Extracellular fluid compartments of the body

The body is comprised of 60% water, distributed in different distinct compartments, intracellularly and extracellularly (Fig. 1). These have variable accessibility for samples, possible sample volumes and uses (Table 1).

2.1. Blood

The blood makes up the systemic circulation in the body with a volume of approximately 5 L, containing blood cells, platelets and blood plasma, with the extracellular fluid (plasma) making up approximately 60% of the volume [7] (Fig. 1). It is an easily obtainable, relatively non-invasive sample, and plasma has thus been extensively analyzed for gene, metabolite and protein contents with genomics, metabolomics and proteomics. However, these analyses of plasma are challenging due to the high dynamic range of analyte abundances, as for proteins ($\sim 10^{10}$), with albumin, immunoglobulins, fibrinogen, transferrin, haptoglobin and lipoproteins making up the greatest portion of the total protein weight (> 90%) in plasma [1,29]. Thus, plasma should mainly be used for established clinical tests and targeted validation studies of already identified biomarkers.

2.2. Lymph

Lymphatic vessels extend throughout most of the body, and function by facilitating the clearance of excess fluid, solutes and macromolecules from the interstitium to maintain tissue homeostasis [28,30], with approximately 2.5 L of lymph fluid draining into the blood per day [7]. Lymph vessels are present in most tissues and have recently been identified in the dura mater of the brain [31,32]. The lymphatic system originates in the interstitium as initial (afferent) lymphatic vessels, drains to larger collecting lymph vessels that goes through lymph nodes, and the fluid drains back to the blood.

2.3. Interstitial fluid

The interstitium, or interstitial space, is the space located outside the blood, the lymphatic vessels and the cells. It contains the IF, proteins, solutes, and the ECM. The tissue microenvironment has been extensively reviewed [6,28,33,34] and will not be discussed in detail here. However, it is important to understand how the soluble components of the IF, namely ions, metabolites, signaling molecules and proteins, enter the IF. All tissues are perfused by fluid entering the interstitium across the semipermeable plasma membrane of blood capillaries, and this fluid contains nutrients, signaling substances and proteins from the blood. The compounds present in the tissue-perfusing fluid will distribute in the tissue according to affinity, molecular size and charge. In addition, the cells of the tissue, both organ-specific and ECM related cells (*e.g.* fibroblasts and dendritic cells) produce and secrete substances to the IF. These can act in the interstitium, as well as being transported with the lymph vessels to lymph nodes and further back into the blood stream [28,34]. In tumors, with non-functional lymph vessels and leaky capillary membranes, IF may also exchange directly into the blood.

The volume of the interstitial fluid in the different tissues of the body vary greatly, from 10% of wet tissue weight in skeletal muscle to 50% in skin and even higher in pathological conditions, with IF making up 60% of wet tissue weight in tumor tissues [28]. Approximately 20% of our body weight comprise of extracellular fluids, of which 15% is located in the interstitium (with plasma comprising the last 5%) [7] (Fig. 1).

Table 1
Overview of extracellular fluid compartments with examples of available sampling volumes, invasiveness of sampling, complexity of sample, proximity to disease and main uses.

Fluid	Invasiveness	Available volume	Complexity	Proximity to disease	Use
Serum/ Plasma	*	> 10 mL	***	Global	Clinical evaluation/diagnosis/validation studies
Cerebro-spinal fluid	**	5–10 mL [25]	**	Enriched	Clinical evaluation/diagnosis/biomarker discovery
Prenodal lymph	**(*)	2.3 μL/min [26], 0.4–3.3 mL/h [27]	*	Local	Biomedical research and biomarker discovery
Interstitial fluid	***	30–43 μL/g tissue ([3]; [28])	*	Local	Biomedical research and biomarker discovery

* = Low, ** = Medium, *** = High.

2.4. Brain interstitial fluid and cerebrospinal fluid

The interstitial space of the brain is supplied with nutrients and proteins from the blood through the strictly controlled blood-brain barrier. In addition, the blood-cerebrospinal fluid (CSF) barrier provides transport of metabolic waste, proteins and signaling substances from the brain to the CSF through the choroid plexus (from peripheral blood) and arachnoid membrane (from cerebral blood). The total volume of CSF is a small fraction of the total body fluid compartments (approximately 150 mL), but it is a tightly regulated compartment that have received much focus. Approximately 20% of the proteins in CSF originate from the brain IF, and thus provide an enriched sample representing physiological processes in the brain [35,36]. The CSF has long been thought to exclusively drain to venous circulation through arachnoid granulations. However, the recently discovered glymphatic system that transports interstitial fluid from the brain to the CSF, and functional lymph vessels in the dura mater that transport fluid from the CSF to the deep cervical lymph nodes, demonstrate alternative routes for transport from the brain and into the systemic circulation [31,32] with implications for transport calculations of the brain. The sampling of CSF is usually done by a lumbar puncture, and this provides access to a fluid containing substances from blood (transported across the blood-brain barrier) and substances released by the central nervous system and brain. In addition to neurotransmitters, proteins have been extensively analyzed in CSF, *e.g.* in the search for biomarkers of neurodegenerative diseases [37] and cancer [25].

2.5. Methods for isolation of IF

To be able to study proteins and other substances in the microenvironment in a quantitative manner, IF needs to be isolated. IF is not readily available, but various methods have been used for isolation of IF, for various analytical purposes. These have been described and evaluated in detail in earlier reviews (*e.g.* [28]). Here, we will consider the main methods by applicability of the isolated IF for quantification of the tissue-to-blood gradient for various substances (Fig. 2). A central question is whether the isolated fluid is native or derived, if the recovery of the substances of interest is sufficient, and if the measured concentrations of interesting substances can be related back to *in vivo* tissue concentration. The methods are summarized in Fig. 2 and Table 2.

2.6. Lymph

Even though initial lymph cannot be defined as IF *per se*, it is generally accepted that IF and lymph have the same composition, and that IF and prenodal lymph are equal representatives of the microenvironment for cells in normal tissues [26,38]. However, the lymph vessels need to be cannulated in initial lymph vessels before collecting lymph nodes, and under steady-state conditions [28]. By isolating afferent lymph from the leg of human subjects, Nanjee et al. was able to compare the composition of lipoproteins in plasma and IF, and quantify cholestrol uptake in high density lipoproteins in IF [27]. The lymph flow, and thus sample volumes, can vary substantially. Semaeva et al. [26] were able to isolate 2.3 μL/min of prenodal lymph from the spleen of rats (collection period lasted for 3–4 h), while Nanjee et al. [27] isolated 0.4–3.3 mL lymph per hour from afferent lymph vessels in the leg of human subjects. Tumor tissue does not contain functional lymph vessels, thus making the technique inapplicable for TIF isolation.

2.7. Tissue centrifugation

The method for isolating IF from tissue by centrifugation was initially developed for cell-poor and collagen-rich tissues like cornea [39] and tail tendon of rats [40] but have later been transferred to other tissues, such as mammary tumors [41], and ovarian [11] and

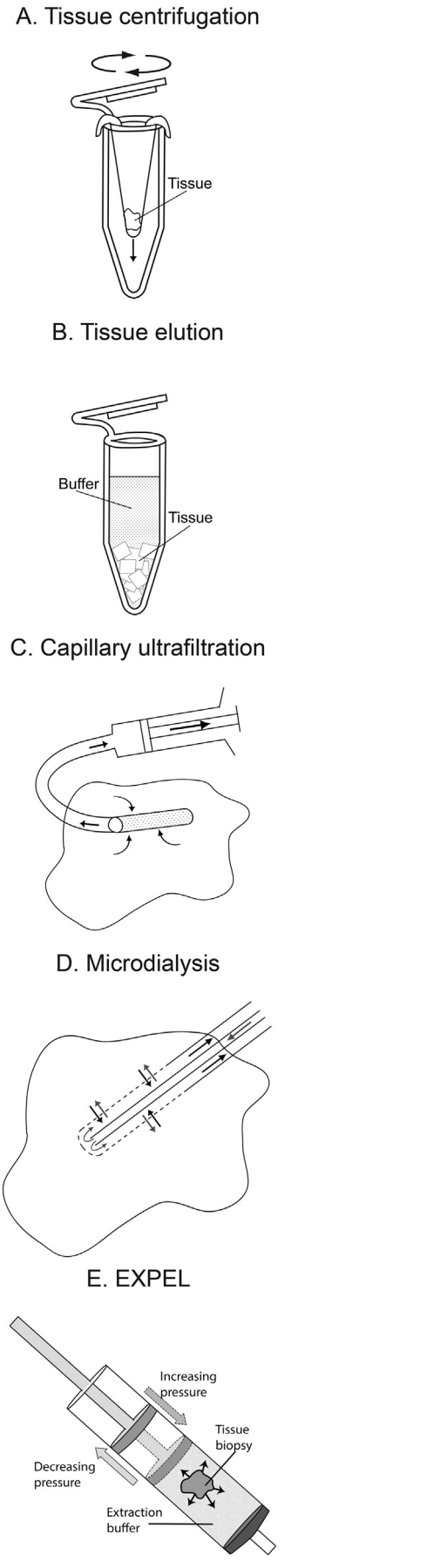

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Fig. 2. Schematic illustration of isolation techniques used for isolation of interstitial fluid that are discussed in more detail in the review. Namely A) Tissue centrifugation, B) Tissue elution, C) capillary ultrafiltration, D) microdialysis and E) Pressure-assisted extrusion (EXPEL), modified from [6,52].

endometrial carcinomas [42]. The principle of the method is that excised tissue is placed on a nylon mesh and centrifuged carefully. The fluid that accumulates in the bottom of the sample tube is assumed to be IF. Two major factors are important for the use of this fluid as native and undiluted IF for quantitative analyses. First, the evaporation from the tissue surface before centrifugation must be controlled. Drying of the tissue can result in an increased concentration compared to *in vivo*, and the isolated tissue should be handled in a humid atmosphere [11] to avoid such evaporation. Second, results show that exposing the cells to high G-forces can lead to rupture of cell membranes, and the admixture of intracellular fluid to the isolated sample [36]. By using endogenous (Na^+ and creatinine) or added tracers (^{51}Cr-EDTA), predominantly present outside the cells, intracellular fluid admixture can be assessed [21]. This demonstrated that true IF could be isolated from rat mammary tumors (< 424 G) [41] and ovarian healthy tissue and carcinomas (< 104 G) [3,11]. However, endometrial carcinomas demonstrated lower Na^+-ratio and likely intracellular admixture at 104 G [3]. Thus, any new tissue type applied for centrifugation should be carefully considered, and special care should be given to fragile or highly vascularized tissues. The amount of IF that can be isolated ranges from a few microliters in skin samples from mice, to larger volumes from hydrated tumors. As an example, a median amount of 75 μL TIF was isolated per gram wet weight tissue from 42 human ovarian tumors (own unpublished data). The centrifugation technique has been used to quantify proteins [43] and small molecules [44].

2.8. Tissue elution

The tissue elution method was first developed by Celis et al. in 2004 [45]. Briefly, a tissue piece (0.2–0.3 g) is cut into small pieces and incubated in PBS for 1 h at 37 °C. This method have received widespread use for qualitative proteomic analyses [14–16], and now recently also for miRNA analysis [46]. The method provides derived IF, where substances from the microenvironment are diluted in PBS buffer, and are dependent on the diffusion of substances from the tissue for the quite short time frame of one hour. Earlier work have shown that proteins are equilibrated in PBS after 48 h [47], using solid tumor pieces, and thus mincing of the tissue is likely necessary to recover proteins in only one hour. However, to my knowledge, actual recovery of proteins using this technique have not been evaluated by the original authors, or any other users of the method. Cutting of cell-rich tissue, like tumors, can yield fluid with a substantial admixture of intracellular fluid [48], making the origin of the identified proteins in such a derived TIF fluid challenging to determine.

Considering these points, it is unlikely that IF from tissue elution can be used to quantify the exact concentration of substances in the microenvironment, and hence, the tissue-blood gradient. However, methodological adjustments can make the information available. First, the exact weight of the tissue added to an exact volume of PBS must be known. To avoid problems with evaporation, the mincing of tissue should be done in a humid atmosphere, and the tissue mass must be determined before adding PBS. Second, the extracellular volume of the tissue must be calculated [3] or assumed based on previous data (see Tables 1 and 2 in [28]). Additional controls should be considered, to account for whether the diffusion of proteins into the buffer is complete and if the IF is contaminated with intracellular fluid and protein load. Such experiments have earlier been performed with rats and the use of extracellular radiolabelled tracers [49]. The Na^+-method is not applicable for elution in PBS, as the buffer in itself contributes substantial Na^+. Using a Na^+-free phosphate buffer is possible, but will likely cause increased rupture of cells due to ionic imbalance [50], and yield suboptimal elution conditions. Monitoring the protein concentration in the extracted IF related to added wet weight of tissue could provide indications on whether the diffusion is sufficient. In addition, as the intracellular protein concentration is much higher than extracellular [51], a high protein concentration may indicate disintegrating cells. Finally, the degradation of protein and other substances may be substantial with incubation at 37 °C even for one hour. The addition of protease inhibitors could be considered.

2.9. Pressure-assisted IF extrusion

Recently, a novel technique, named EXPEL, based on rapid, pressure-assisted IF extrusion was demonstrated, focusing on the isolation of IF from clinical specimens while preserving the specimen for subsequent routine clinical pathology investigation [52]. Briefly, fresh surgical biopsies are cut in 3 mm^3 pieces and placed in hypertonic buffer inside a plastic syringe. The pressure is then alternated three times (between 0.8 and 1.2 bars) by moving the syringe plunger. The resulting buffer is moved forward as IF, while the tissue re-entered the classical tissue processing for clinical evaluation. The method yields a sample suitable for comprehensive analyses of proteins, metabolites, miRNAs and DNA, and also demonstrates the presence of exosomes. The strengths of the methods are thus the rapid preparation of only

Table 2
Overview of isolation methods for interstitial fluid, the expected volumes isolated, the ability to use the isolated fluid for absolute quantification and the applicability for different isolated substances.

Method	Dilution	Isolated volume (μL)	Time for isolation	Quantity	Applicable for analysis of					Reference (examples)
					Nanoparticles/ extracellular vesicles	Proteins	Meta-bolites	Nucleic acids	Small compounds	
Microdialysis	Y	10 μL/6 h	6 h	N	X	X	**	*	**	([13]; [57])
Capillary ultrafiltration	Y	0.05–10 μL/min	6 h	N	X	X	**	*	**	([60]; [61]; [62])
Tissue elution	Y	0.8 mL	1 h	I	**	**	**	**	**	([14]; [45])
EXPEL	Y	1 mL	3 min	I	**	**	**	**	**	[52]
Tissue centrifugation	N	30–43 μL/g tissue	10 min	Y	**	**	**	**	**	([11]; [48])
Lymph	N	2.3 μL/min (rat spleen), 0.4–3.3 mL/h (human leg)	3 h	Y	*	**	**	**	**	([26]; [27]; [38])
Open-flow microcirculation	Y	NA	8 h	N	X	*	**	*	**	[65]
Vacuum-based techniques	N/Y	100–250 μL/2 h (not diluted)	2 h	N	X	X	**	*	**	([49]; [68]; [69])

Y = Yes, N = No, I = Indirect, X = not suitable, * = suitable, ** = very suitable.

minutes, and that the same sample can be used for IF isolation and clinical assessment [52,53]. However, the method is, as the elution method, based on diluting the IF in an unknown weight-volume ratio in buffer. The absolute information about concentration of the various components analyzed in IF is not readily available. Weighing of the tissue and buffer can be possible, and a comparison with protein yielded from the extraction and into the isolated buffer may provide some information about quantity. It remains to be seen if the method can be included in the pipeline for routine clinical investigations.

2.10. Microdialysis

Microdialysis is a catheter-based *in vivo* sampling method frequently used to sample small molecular substances from the interstitial space of various tissues, such as brain, muscle, skin and tumors [54–57]. A semipermeable membrane on the tip of a catheter is placed within the tissue of interest by a cannula and the probe is continuously perfused with a physiological solution at low flow rates (*e.g.* 1 μL/min). Analytes in the interstitial space will diffuse into the buffer solution, and can be recovered through sampling of the efflux solution. The diffusion will depend on membrane permeability, perfusate flow and area of the membrane [54]. Recovery for small molecular weight drugs *in vitro* can be high (> 90%), while the recovery in tissue tend to be lower (69, 50 and 29% for blood, muscle and brain respectively) [56]. This discrepancy is caused by the sampling means *in vivo* and *in vitro*. For *in vitro* determination of recovery, the microdialysis probe is placed in a physiological buffer containing the substance of interest, and the primary factor limiting diffusion into the probe will be the resistance to diffusion of the semipermeable membrane. On the other hand, *in vivo* sampling happens within the tissue, and diffusion will primarily be limited by tissue resistance to drug diffusion. In addition, *in vivo* recovery rates are highly variable (%CV > 20), and recovery of *in vivo* probes should be made for each probe in every experiment [56]. For cytokines, recovery of 1–3% has been reported *in vivo*, based on tissue homogenate concentration [58]. Fluid isolated with microdialysis have been applied to qualitative metabolomic [57] and proteomic [13] studies. Microdialysis have also been applied to studies of drug pharmacokinetics and pharmacodynamics, which both are dependent on quantitative information [54]. Important to note, to obtain “true” tissue concentrations, the microdialysis probe must be calibrated by exogenous or endogenous substances of known concentrations and the diffusion through the tissue must be high enough to obtain a steady-state in the area of the probe [59]. Nevertheless, measurements are hampered by low recovery, and that steady-state in many cases cannot be established. The use of a generic standard to calibrate the probe will have limited application, as the recovery vary substantially for analytes of different sizes, as well as over time [59]. A tissue concentration reference can be acquired by tissue homogenization, but for all locally produced substances, the intracellular presence of the substance will influence the concentration measured.

2.11. Capillary ultrafiltration

Another option for IF isolation is to use implanted capillary ultrafiltration probes and applying negative pressure to recover solutes from the interstitium by bulk flow [60–62]. The cut-off of the semi-permeable membranes used will affect the recovery of molecules, and a cut-off of 400 kDa is frequently used to allow for passage of large proteins. *In vitro* experiments have demonstrated close to 100% recovery for small proteins (*e.g.* insulin), and 50–100% recovery for albumin [63]. However, the protein concentration measured in isolated fluid from mouse fibrosarcomas [64] was calculated to be 1/2000 of the protein concentration measured from IF isolated by other approaches in other mouse tumors [28]. This indicates that the recovery of large molecules *in vivo* is indeed poor, and the protein composition in the isolated fluid is not comparable to *in vivo* composition of large macromolecules. The great advantage of this method is that *in vivo* fluid sampling over time is possible, and may yield small molecules, metabolites and possibly microRNA in relevant concentrations in isolated fluid not diluted with buffer, retaining quantitative information. The draw-back is that the method collects minute amounts of fluid, *e.g.* 10 μL of fluid collected in 6 h [64], with inherent risks of sample loss, degradation and adsorption issues due to small volumes.

2.12. Open-flow microcirculation

While the membranes used in ultrafiltration and microdialysis techniques restrict the recovery of large molecules, open-flow microperfusion is based on a probe with macroscopic perforations (*e.g.* diameter of 0.3 mm) allowing direct access to IF [65] enabling sampling of compounds as albumin [66] and insulin [67]. The recovery of substances in the perfusate will however still be dependent on the same factors as microdialysis, *i.e.* size of the exchange area, perfusion flow rate, and tissue resistance to diffusion, and additional steps must be taken to be able to acquire quantitative data. By recirculating the perfusate and using sensors for specific analytes in the sampling probe, Schaupp et al. proposed a new approach to quantify interstitial analytes *in vivo* [65]. However, the method has only been evaluated for measuring electric conductivity in the IF, and should be extended to defined analytes to be able to assess the applicability for measuring true interstitial concentrations. A main drawback of the method is the need for a specific sensor to monitor each analyte of interest. This will likely limit the use of such quantification methods for many substances.

2.13. Vacuum-based techniques

The suction blister technique has long been applied for the analysis of analytes from the skin interstitium [28], and these techniques can include isolation of undiluted fluid or using perfusion with a buffer to isolate the samples. Similar to ultrafiltration, the applied negative pressure will cause hyperfiltration, and reduced recovery of large molecules. In recent years, ultrasound has also been used to permeate the stratum corneum before using a vacuum chamber to isolate fluid from the skin interstitium to quantify lipoproteins [68], isolate samples for proteomics [69] or monitor insulin levels [49]. The reported concentrations of lipoproteins were 18–25% that of blood [68], while the protein concentration measured by Lecomte et al. [69] averaged at 1.3 μg/μL, or 1/56 the concentration found in human blood [70]. Thus, although improving on ultrafiltration, the macromolecular recovery is still low and not representative for true IF.

2.14. Invasiveness – and access to samples

In addition to the quantitative information that is obtainable by different IF isolation methods, there are variable levels of invasiveness, that will determine the applicability of a certain method. The methods can be divided in *in vivo* and *ex vivo* methods.

The collection of lymph is an *in vivo* technique, but requires surgery with exposure of primary lymph vessels and prolonged collection periods, and has its prime area of use in animal experiments, or in skin in human subjects. Capillary ultrafiltration and microdialysis can both provide *in vivo* collection of fluid from the interstitium in humans without the need for excising tissue or extensive surgery – and has increased applicability in (patho)physiological conditions not requiring removal of tissue from the body, but where monitoring the extracellular microenvironment is attractive. Microdialysis has demonstrated safe use when implanted intracranial for up to 5 days, providing the possibility to monitor biochemical changes in the extracellular space over time [54]. However, the sample volumes of ultrafiltration and microdialysis are very small, limiting the down-stream processing of the samples. Tissue centrifugation, tissue elution and pressure-assisted extrusion all need excised tissue requiring surgery. Depending on the

analytes of interest, pressure-assisted extrusion could also be used on biopsies, if further clinical evaluation of the same tissue can be ensured. Because the tissue is processed *ex vivo* by these methods, the surgical time or exposure to the patient is not altered, making the methods applicable for human subjects especially in relation to cancer, but also other conditions where tissue is surgically removed.

3. Measuring gradients in fluid

The ability to measure the concentration of an analyte in both tissue interstitium and other fluid compartments, *e.g.* lymph, blood or CSF, will provide data for determination of mass transport of analytes and steady-state conditions that cells experience, as well as indicate the origin of a substance. By being able to assess whether a substance is present by passive diffusion from plasma, secretion from cells in the tissue, or homing from other tissues or external sources (*i.e.* therapeutics) we can obtain better knowledge on various physiological processes and evaluate tissue uptake of therapeutic agents.

3.1. Tissue-originating substances

Plasma harbors important information for understanding the relevance and origin of analytes in the microenvironment [2]. Since any solute transported across the microvasculature from plasma will be present in a lower concentration in IF than in plasma, an IF-to-plasma ratio > 1.0 shows local production [2,6]. The measure of this ratio relies on IF isolated without damaging cells of the tissue, because cell rupture with the subsequent admixture of intracellular analytes will occlude such conclusions. As examples, Nedrebø et al. [71] quantified the pro-inflammatory cytokine TNF-α in IF and plasma and showed that TNF-α was produced locally in skin during ischemia-reperfusion, but originated from plasma in endotoxemia. Similar approaches have been applied to demonstrate local production of cytokines in rheumatoid arthritis [72], secretion of peptides by adipose tissue [73] and a correlation between locally produced TNF-α in bone marrow IF and remission status of acute myeloid leukemia [74]. Gore et al. [75] analyzed phenylalanine transport from interstitial fluid to the circulation in septic patients and normal volunteers to assess muscle protein kinetics. All of the examples show how the quantification of locally originating substances and the exchange with circulation can shed new light on biological processes. The experimental values can further be combined with mathematical models on the distribution of cell-released and ECM-released substances in the interstitium [76].

Using IF as a source for proteomic biomarker studies can yield quantitative information suited to highlight proteins likely originating in the tissue of interest, if such quantitative information is preserved during IF collection. The concentration gradient of CA-125 has been demonstrated in ovarian TIF [2] and cyst fluid [77], and VEGF has been quantified [2] and estimated [78] in TIF. Although the protein gradient between tissue and plasma is estimated to be approximately 1000-fold, the actual gradient may be highly dependent on clinical parameters. For example, the tumor-plasma gradient of CA-125 was shown to correlate with tumor stage, indicative of a specific local production of CA-125 in the tumor [2]. On the contrary, the same study showed that VEGF and osteopontin, both cancer-related proteins, had a much lower gradient between tumor and plasma, not dependent on tumor stage, in the same patients. This indicated a much lower specificity and a wider distribution and production of these proteins. The calculation of the individual tumor-to-plasma gradient thus made it possible to highlight a protein with tumor-specific behaviour, and such analyses may help to select more physiological relevant biomarker candidates in future biomarker discovery studies.

IF validated as undiluted by intracellular admixture will contain high concentrations of locally produced proteins that are secreted into the extracellular environment, compared to other tissues and plasma. Using quantitative IF data as an inclusion criterion may identify novel biomarkers with a higher likelihood of proceeding into clinical validation, because such candidates can demonstrate a quantitative relevant presence in target tissues compared to controls – and not a mere detection *versus* non-detection often applied for biomarker candidate selection [21,22,24].

3.2. Plasma-originating substances

Molecules not produced in the tissue but transported there from the circulation will experience the phenomenon of volume exclusion [79] caused by ECM molecules as collagen and glucosaminoglycans, restricting the space available for soluble macromolecules, simply because molecules cannot occupy the same space. This is particularly relevant for large molecules as proteins. The experimental exclusion properties of a molecule can only be determined by access to undiluted IF samples. To calculate a molecule's tissue distribution properties, the concentration of the molecule in the available fluid compartment can be measured in isolated, undiluted IF, and the total amount of the molecule can be measured in the supernatant from prolonged tissue elution (where the extracellular volume and supernatant is equilibrated). The available volume can then be calculated as V(av) = mass/concentration. To assess how much of the extracellular volume of the tissue that is actual available for the analyte of interest, the available volume of the analyte is compared to the available volume for a small molecule with predominant extracellular presence (*e.g.* Na^+, creatinine or ^{51}Cr-EDTA) [3,80].

The volume exclusion phenomenon exemplifies why access to true IF is important. Methods isolating diluted IF or using whole tissue homogenates, to quantify the presence of interesting substances, do not take into account the extracellular volume of the tissue or the possible exclusion of the substance from parts of that extracellular volume. Thus, difference in quantity in derived IF can be caused by difference in extracellular volumes in the studied tissues, rather than true differences in extracellular abundance. For therapeutic agents this may have particular relevance.

3.3. Therapeutic agents and nanoparticles

In addition, IF can be a powerful tool for the characterization of tissue uptake and distribution of therapeutic agents [79], and relate this to drug concentration in the circulation. Such data is central for development of novel drugs and improved targeted therapies. Diffusion of a substance in tissue is determined by various properties, including its molecular size and shape, its solubility in water and lipids, the composition and structure of the ECM and by the metabolism and binding to proteins and tissue components for the substance [81] (Fig. 3). This has been demonstrated experimentally for anti-interleukin 17 IgG by Eigenmann et al. [82,83], and they found that antibody interstitial concentrations are both highly tissue-specific, but also dependent on the underlying capillary structure. They showed that the measured interstitial concentrations of antibodies in muscle and skin were surprisingly high, at 50–60% of the vascular concentration. Furthermore, the interstitial concentrations were > 10 times and > 3 times higher than total tissue concentrations for muscle and skin, respectively. Such analyses and comparisons clearly demonstrate the advantage of sampling and analyzing the interstitial fluid as a separate fluid compartment.

This may be particularly important in relation to anti-cancer drugs. To kill a high proportion of tumor cells in a solid tumor, the cytotoxic agent needs to distribute through the tumor vasculature, cross vessel walls and transverse the tumor tissue. However, the heterogeneous tumor microenvironment often results in marked gradients in drug concentration, resulting in poor therapeutic effect and development of drug resistance [81].

In vitro (spheroids and multi-layered cell cultures) and *in vivo* (window chamber) methods have been used to examine drug

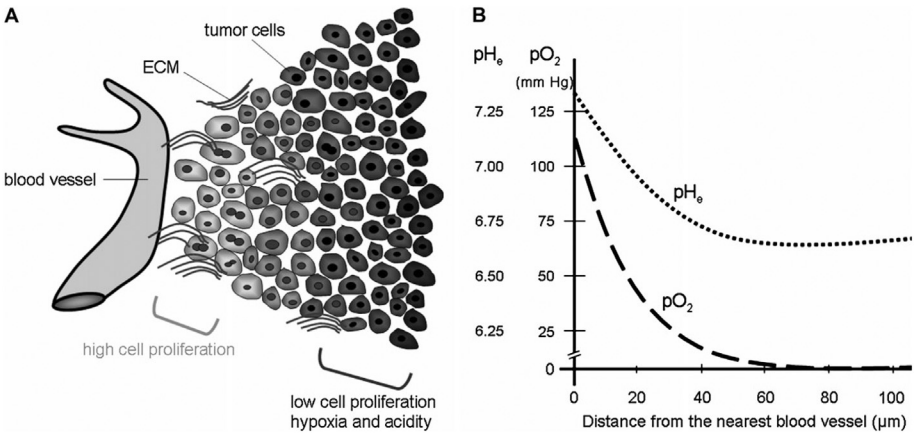

Fig. 3. The tumor microenvironment in relation to blood vessels. A) Diagrammatic representation of tumor cells and the extracellular matrix (ECM) surrounding a capillary. B) Schematic representation of the gradient of oxygen concentration (pO2: dashed line) and of pH (dotted line) in relation to the nearest tumor blood vessel. From [81].

penetration and distribution in tumors, *e.g.* by fluorescence microscopy and autoradiography [81]. An alternative to *in vivo* methods is to quantify the concentration of agents in tissue sections by histology and imaging. These will provide position data, but direct quantification can be challenging due to the need for labelling of the interesting compounds. Also, immunohistochemistry of excised tissues will provide good detail on intracellular presence but will usually not retain information about soluble factors in the extracellular space. An alternative is mass spectrometry-based imaging, where laser ionization of discrete points is used to obtain spatial distribution data for specific substances, recently also demonstrated in a quantitative fashion [84,85].

Quantification of compounds in IF may also shed light on extracellular tissue distribution of drugs, however, this is highly dependent on the chosen IF isolation method. Pharmacodynamic and pharmacokinetic studies of therapeutic agents have historically used analyses of drug concentrations in plasma or CSF to assess distribution. However, these measurements do not necessarily reflect the concentrations within the interstitial space. Tissue homogenates similarly cannot provide information about the distribution of drugs in the interstitial space of a tissue [54].

For small molecules, ultrafiltration and microdialysis can represent valuable alternatives to determine uptake in tissue [55], also *in vivo*. For larger molecules (even cytokines) the extracellular concentration will only be accessible by lymph or tissue centrifugation, or alternatively by elution and pressure-assisted extrusion if extracellular volume and intracellular admixture parameters are known.

To obtain quantitative information about tissue distribution a combined method of tissue centrifugation and tissue elution is needed. Then exclusion of macromolecules can be calculated as for plasma proteins and provide quantitative data on tissue distribution after administration of drugs.

Exosomes and nanoparticles [86] are ranging from the size of large proteins and upwards, and can only be recovered by tissue elution, pressure-assisted extrusion, tissue centrifugation or in lymph.

4. Shortcomings and needs for further method development

The inconvenient truth about cancer biomarkers, presented by Tadashi Kondo in 2014 still holds true: *none of the reported protein biomarkers have proven to be beneficial for cancer patients* [22]. It seems that regardless of the biomarkers being DNA, RNA, protein or metabolite based, no biomarkers are able to advance from the first discovery to the clinic [21]. Despite the great advances in -omics technologies, there remain large problems in study design, sample selection and the consideration of disease tissue heterogeneity, that is: linking the -omics data to physiological processes and compartments of the body. In cancerous tumors you will find heterogenous cell types, hydration and structural variations of the ECM that will all influence composition and identification of extracellular substances. In addition, the tumor will not likely produce any unique proteins, most proteins are found in the entire body [24] – in various amounts. Thus, the identification of central biological processes and important export products of a tumor will always rely heavily on the ability to accurately quantify these substances. As detailed in this review, such quantification is highly dependent on the choice of samples and sampling procedures. By utilizing one or several of the methods described in literature in-depth quantitative analysis is possible, providing that the necessary controls and quantitative assessments are being made as the samples are collected. The integration of IF sample collection in the clinical assessment of excised tissues as suggested for the EXPEL method may even enable us to review larger cohorts of patient samples in a quantitative way, to take the next step into quantitative evaluation of the tissue microenvironment and the fluid gradients of the body.

5. Conclusion

There is increasing focus on the tissue microenvironment and tissue interstitial fluid in medical research today, especially linked to cancer research. However, the majority of studies of the interstitium are of a qualitative character, not encompassing the quantitative data that can be found in the interstitial fluid. There is a need for more focus on quantitative measures to integrate functional knowledge of IF and the extracellular environment with the surge of new studies on the transcriptome, metabolome and proteome for various healthy and disease states in human physiology. A reflected choice of IF isolation methods and analyses can further contribute with quantitative measures for drug distribution in tissue as well as provide clinically relevant data on the concentration gradient of substances to and from the circulation. Ultimately, this may lead to new insight for physiological processes, as well as diagnosis and treatment of diseases, such as cancer.

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