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Lab-on-a-Chip hyphenation with mass spectrometry: strategies for bioanalytical applications

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The Lab-on-a-Chip concept aims at miniaturizing laboratory processes to enable automation and/or parallelization via microfluidic chips that are capable of handling minute sample volumes. Mass spectrometry is nowadays the detection method of choice, because of its selectivity, sensitivity and wide application range. We review the most interesting examples over the last two-and-a-half years where the two techniques were used for bioanalytical applications. Furthermore, we discuss the merits and limitations of such hyphenated systems. We inventorize the reported applications and approaches. We see an ongoing trend towards chip-based liquid chromatography–mass spectrometry usage and small volume analysis applications, particularly in the field of proteomics where bottom-up approaches profit from chip-based technologies and hyphenation with complex cell cultures.

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Introduction

Current day challenges in bioanalysis include automation, throughput, small volume handling and disposability. These challenges are addressed with increasing success through miniaturized laboratory processes, so-called Lab-on-a-Chip (LOC). LOC systems use microfluidics (see Supporting information) to handle minute volumes and can be manufactured as cost-effective disposables. Moreover, it is possible to integrate laboratory protocols and/or analysis methods into a single cartridge.

Typical detection techniques that are combined with LOC techniques include optical detection (e.g. UV/Vis spectrophotometry), nuclear magnetic resonance, and electrochemical detection [1]. To date, none of these sensing techniques can match the selectivity and sensitivity of mass spectrometry (MS).

LOC systems and MS match particularly well. LOC operates in the small volume domain and provides (limited) separation. MS detection improves when downscaled; it has a high resolving power and is sensitive.

One of the first hyphenated LOC-MS systems was reported by Xue *et al.* [2] At the time a key driver for this combination was to increase throughput in diagnostics and screening applications by using multiple fluidic channels and ionization sprays. The authors also perceived the potential for sample preparation, capillary electrophoresis (CE) and liquid chromatography (LC) on chip coupled to MS.

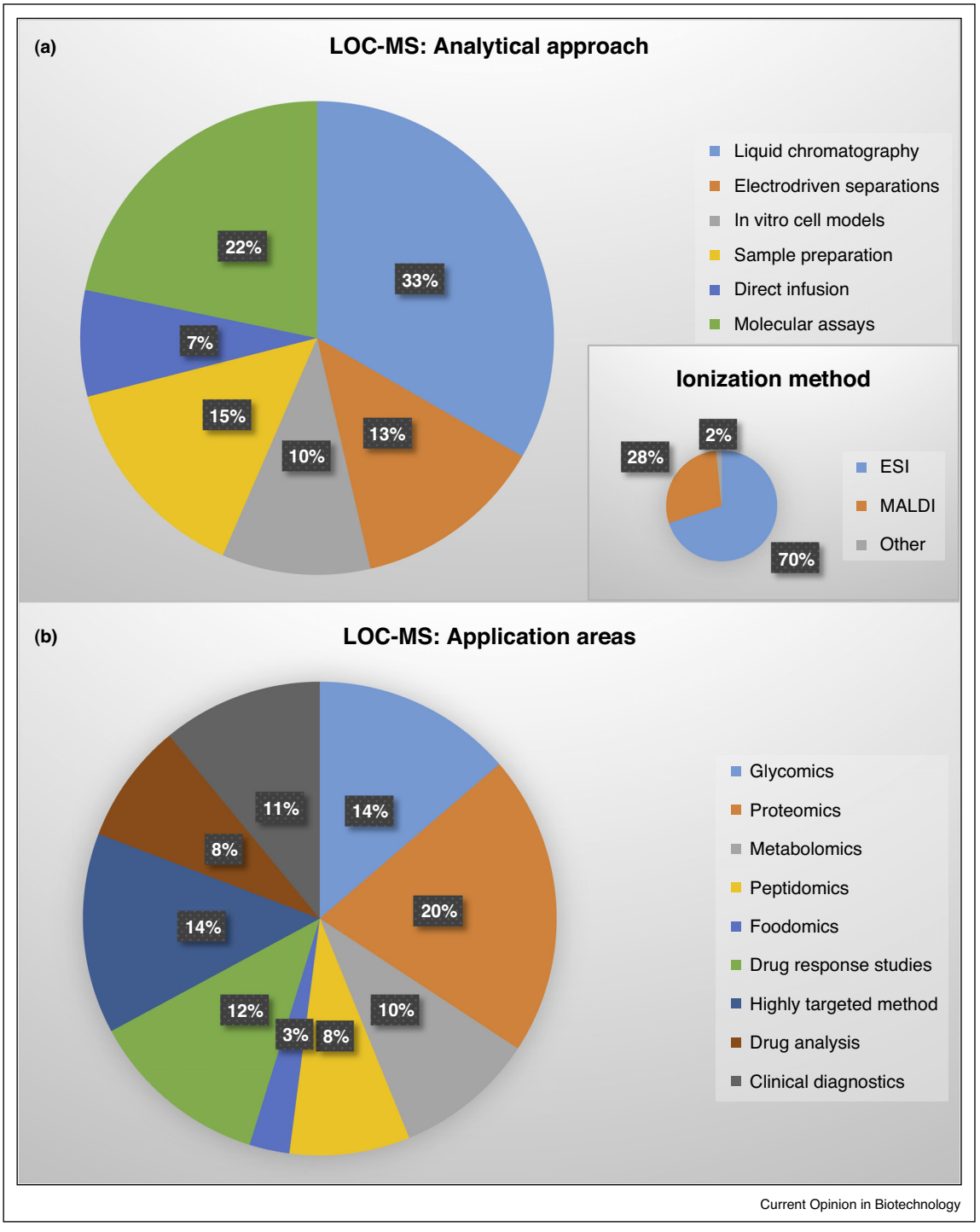
We inventorized developments in the last 2.5 years in the LOC-MS field from two perspectives: analytical approach (Figure 1a) and application area (Figure 1b). The most commonly used approach is LC and the most commonly used application area is proteomics. The review is structured on approaches to sample preparation, direct infusion MS, separation and the total analysis system principle. Comprehensive reviews on LOC-MS have recently been published by Gao *et al.* [3••] and Feng *et al.* [4••]. In this critical review we argue that the combination of LOC and MS will prove to be the ideal combination for bioanalytical applications and we discuss the, in our view, crucial steps forward and the most dominant trends.

Sample preparation

Common sample preparation techniques are liquid–liquid extraction and solid-phase extraction; only one example of the latter was reported on LOCs in the last 2.5 years. Solid-phase extraction was integrated with *in vitro* cell culturing and will be discussed later in the review. In bottom-up proteomics proteolysis is an important part of the sample preparation workflow; the majority of LOCs focussed on this.

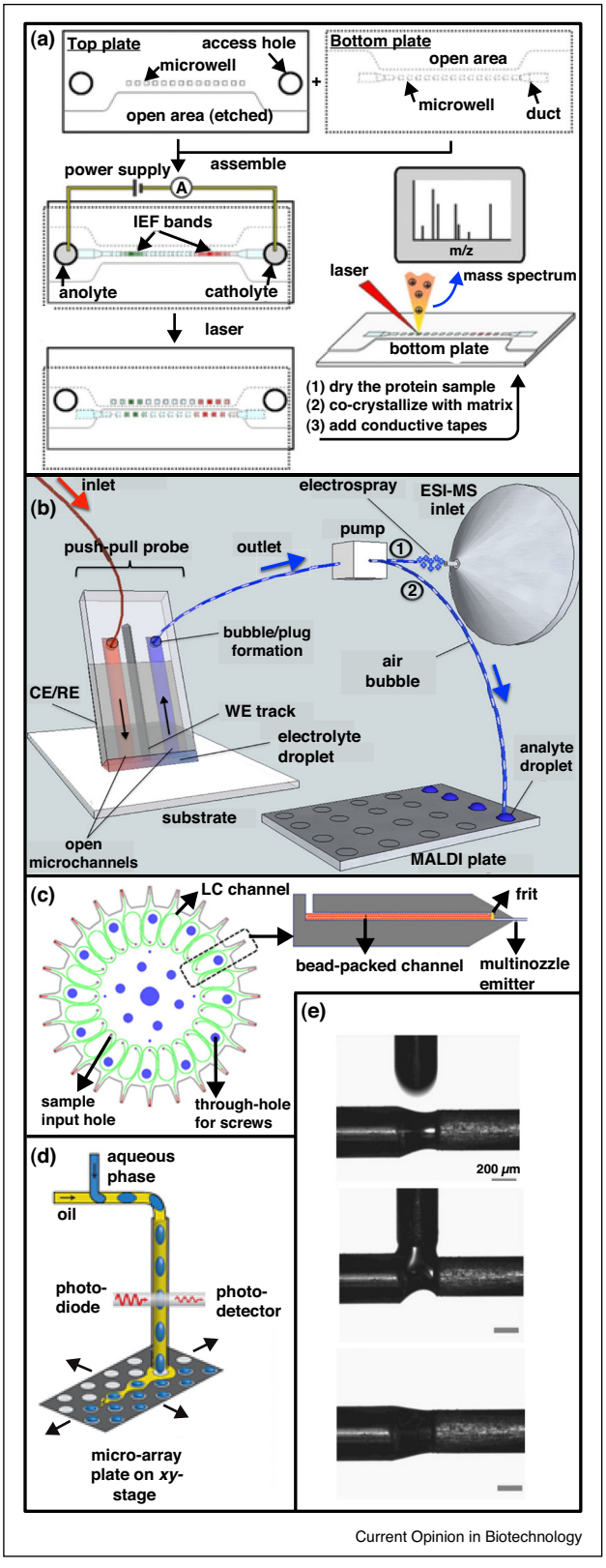
Several devices integrating the proteomics workflow into one LOC were presented. One example is a fully integrated electrowetting-powered LOC capable of automated performance of the whole proteomics workflow

Figure 1



Overview of LOC-MS publications in the last 2.5 years, based on approach and application area of LOC-MS hyphenated techniques. A total of 192 papers published between January 1st 2012 and June 1st 2014 were found using the following query on PubMed ''*((((microfluidic*) OR lab-on-a-chip) AND mass spectromet*)) AND (''2012''[Date - Publication]: ''2014''[Date - Publication])*'', of these 68 were research papers within the scope of this review. These articles were categorized and included in this figure, several articles had multiple classifications. **(a)** A pie chart of published works describing various analytical approaches that were hyphenated to mass spectrometry. The insert provides an overview of the type of ionization sources used. **(b)** A pie chart of research application areas that use LOC-MS.

Figure 2



Developments in hyphenated LOC MS techniques: **(a)** 'SlipChip' based iso-electric focussing hyphenated with MS. The top and bottom chip substrates of each contain microfluidic structures that combined create a microfluidic path. After IEF is performed a slipping operation is performed to disconnect the fluidic path. Separated proteins and metabolites analytes are dried and co-crystallized with MALDI matrix, followed by detection via MALDI-MS. Reprinted with permission [5•]. **(b)** Microfluidic push–pull probe for the scanning of dry surfaces. The push–pull probe immerses the dry sample with an electrolyte and electrochemical measurements are carried out on one nanoliter of electrolyte. The flow is segmented by air bubbles that are introduced at the junction between the probe and the outlet capillary. After that the droplets are spotted on a MALDI plate for analysis or sent to an ESI source. Reprinted with permission [6•]. **(c)** Multinozzle emitter array chip for parallel analyses. The chip consists of 24 channels (which are optionally packed with LC column material) each connected with an emitter. Modified with permission [7•]. **(d)** Droplet microfluidic dispensing interface for MALDI analysis. Droplets are dispensed on a micro-array plate containing hydrophilic (26444) and hydrophobic areas. After deposition the oil phase quickly evaporates. Enzymatic reactions can be monitored over time by spotting droplets and drying them with nitrogen gas followed by deposition of MALDI matrix for MALDI analysis over time. Modified with permission [8•]. (e) Capillary gap sampler for direct analysis of small volume samples. Nanoliters of sample are introduced from a 384-well plate via a liquid junction suspended in between a fused silica buffer supply line and a stainless steel ESI emitter. Modified with permission [9•].

(from sample preparation to acquisition). MALDI was enabled by removing the top cover of the LOC after addition of the MALDI matrix. Then the open LOC was placed into a custom-made MALDI plate and analysis was performed [10]. A device with similar functionality was created using Quake valves to generate and control droplets in an LOC coupled to MS via an integrated nano-ESI emitter [11]. Furthermore, a droplet microarray plate for the proteomics workflow was developed. This microarray was interfaced to ESI-MS via an L-shaped capillary with a tapered tip that served as sampling probe and ESI source [12]. Tryptic digestion for proteomics after LC-based fractionation is normally performed off-line and suffers from low throughput. On-line methodologies involving immobilized trypsin have aspecific adsorption, which leads to carry-over. These problems were solved via an LOC in which LC effluent droplets were trypsinized and consequently quenched. The LOC was interfaced to MS via an integrated stainless steel emitter [13]. Another device interfaced droplet microfluidics with a microarray plate containing hydrophilic and hydrophobic spots for the observation of enzyme kinetics (angiotensin II to angiotensin I conversion) in a massive parallel format — 8265 droplets were deposited on the plate — as shown in Figure 2d — and dried using N_2. Afterwards MALDI matrix was deposited and, because each dried spot represents a time-point, the reaction kinetics could be observed via MALDI-MS [8•]. An LOC with an integrated proteolysis microreactor to monitor hydrogen-deuterium exchange in proteins was coupled to MS via a custom ESI emitter for both continuous [14] and pulse [15] labelling experiments, enabling the characterization of conformational changes that cannot be probed by conventional techniques. In this work, a push–pull probe

was coupled to ESI-MS and a droplet micro-array. This probe was used to analyse dry surfaces via both scanning electrochemical microscopy and droplet deposition on a MALDI plate. It was also used to image immobilized enzymes under a fluid layer by delivering para-aminophenyl phosphate via the microfluidic probe and analysing the para-aminophenol products by ESI-MS (see also Figure 2b) [6•]. An application where electrowetting-based LOCS are applied in combination with ESI-MS is dried blood spot (DBS) screening. Succinylacetone in DBS samples was quantified using a fully automated, nine-step analysis on an LOC. Interfacing to MS was achieved via a removable pulled glass capillary emitter nano-ESI source, inserted between the chip substrates. No statistically significant differences (95% confidence interval) were found between results obtained with conventional methods and the LOC [16].

Direct infusion-MS

Direct infusion-MS (DI-MS) refers to introducing sample into the MS without prior separation. DI-MS chips are marketed by Advion. These systems are capable of delivering robust data, can be used for high-throughput analyses, and utilize disposable tips, thereby removing carry-over. Recently it has been used for ganglioside analysis from the human caudate nucleus [17], DBS alpha-galactosidase assaying to diagnose Fabry's disease [18] and determination of unusual glycosaminoglycans sulphation patterns in murine brain tissue [19]. A competitor for this system is the capillary gap sampler (Figure 2e). Analyte droplets are introduced from a 384-well plate into a liquid junction between a glass capillary supply line and ESI needle [9•]. The main advantage of this system is its ability to sample nanoliters, which is a three-order magnitude improvement over the Advion system.

Separation

Chip-based liquid-chromatography

The vast majority of papers reporting the use of chip-based LC (chipLC) utilized commercial systems, for example from Agilent Technologies, Waters Corporation and Eksigent. Miniaturized LC–MS exists in a variant known as nanoLC–MS, which provides extremely sensitive analysis, but can have issues with robustness due to dead volumes and leaks. ChipLC offers a solution for these challenges.

ChipLC interfacing to MS is often achieved via tubing to ESI sources or on-chip integrated sprayers coupled to a (special) interface. Monolithically integrated sprayers are also emerging [20]. ChipLC–MS has been used for, amongst others: pharmacokinetic studies in rodents [21–23], detecting aflatoxins in peanut products [24], detecting gangliosides and sulfatides in murine brains [25], glycomic [26] and proteomic biomarker discovery [27], quantification of matrix metalloprotease-9 in bronchoalveolar lavage fluid [28] and quantification of stable isotope dimethyl-labelled phosphopeptides [29]. ChipLC–MS steroid analysis [30,31] demonstrated improved LOD than conventional LC–MS. ChipLC was also coupled to MALDI-MS, using EOF-based pumps. After separation the proteins were transported orthogonally via electroosmosis in microchannels to MALDI reservoirs [32]. Another important development is that, to our knowledge, chipLC–MS was used for the first time on patient samples in a phase II clinical trial. ChipLC–MS was used to monitor incorporation of deuterated leucine into an apolipoprotein(a)-derived peptide [33]. This indicates that ChipLC–MS is currently at a level of robustness that pharmaceutical companies are willing to employ it during drug development.

Other significant developments indicate maturation of chipLC–MS are the appearance of validated chipLC–MS methods for analysis of illegal drugs [34], monitoring of fluoxetine and norfluoxetine in rat serum [21] and 7-ethy-10-hydroxycampotothecin in murine plasma [22]. A commercial application by Newomics Inc. is the multinozzle emitter array chip (Figure 2c), which can be used for parallel DI protein analysis and enhanced throughput chipLC–MS analysis of tryptic digests, thanks to the sensitivity enabled by the multiple nozzles per emitter [7•].

Electro-driven separations

The main challenge in chip-based electrodriven separation systems lies in MS interfacing. Recent chip-based capillary electrophoresis (chipCE) works have focused on increasing the robustness of interfacing to MS, for example through monolithic integration of ESI tips [35,36]. Also, an integrated make-up flow chip design and its effect on separation, LOD and robustness of amino acid analysis was demonstrated [37]. Furthermore, chips utilizing zero, one and three make-up flows were compared. The authors conclude that, while LODs for cardiac drugs are improved without make-up flow, the LOCs with make-up flow are more robust and easier optimized [38].

Optimal chipCE–MS conditions for proteins and peptides are challenging: a low ionic strength background electrolyte and acidic pH are required for efficient ESI. Under these conditions silica is prone to electro-osmotic flow (EOF) instability due to protein–wall interactions. Batz *et al.* coated silica channel walls with aminopropyl silanes, ensuring stable EOF between pH 2.8 and 7.5, and an inter-device EOF reproducibility of 2.6% RSD. Protein analysis showed 0.7% RSD migration time reproducibility and plate numbers up to 400 000; peptide separation efficiency was over 600 000, the highest reported for any CE–ESI-MS. ESI was achieved from the corner of the chip aided by electroosmosis-driven make-up flow [39•].

In another electro-driven separation, capillary isoelectric focusing (cIEF), ampholytic analytes are separated according to their isoelectric point in a pH gradient. Wang *et al.* developed a methodology to hyphenate chip-IEF to MS using nanowells in glass bottom and top plates, which, when oriented in a semi-overlapping fashion, form a separation channel. After separation the glass plates were moved, resulting in MADI-MS-ready nanowells containing separated analytes. Eleven amine metabolites were putatively identified in CSF using this method [5•].

Li *et al.* integrated cell culturing and chiral chipCE–MS analysis in one LOC. Cell culturing was performed on a 0.22 μm filter on top of the sample inlet channel; downstream the separation channel, chiral selectors (moving opposite to the net flow) were introduced and periodically the extracellular matrix was sampled. ESI took place at corner of the chip, aided by a make-up flow. The enantioselective catabolism of racemic DOPA by neuronal cells was monitored [40], showing that chipCE is a feasible technique for analysis of *in vitro* cell models.

Towards integrated total analysis systems

Hyphenating *in* vitro cell models to MS is attractive as the information level provided by MS exceeds traditional optical detection techniques. Furthermore, on-line analysis allows following kinetics. Several LOC devices integrating biological experiments and sample preparation have been developed by the Jin-Ming Lin group. In these devices, micro-solid phase extraction is integrated. The interfacing to MS is achieved via tubing connected to an ESI needle. Applications include: measuring acetaminophen metabolism via cultured microsomes [41], quantitative analysis of tumor cell metabolism of genistein [42], testing of absorption of various concentrations of methotrexate and its cytotoxic effects [43] and the uptake of curcumin by CaCo2-cell lines [44]. One system was used for studying signalling molecules in cell-cell communications [45]. Emerging trends involving 3D cell culture and organ-on-a-chip will likely increase the attention for these types of systems.

Outlook and conclusion

An overview incentives and pre-requisites for adoption of LOC-MS systems is presented in Table 1. The incentives to use LOC-MS are to enable small volume analysis, high throughput/parallelization and automation, time-continuous monitoring and on-line sample preparation. Several of these pre-requisites have already been fulfilled. Commercialized systems as well as cartridge-integrated set-ups are present especially in the chipLC–MS field.

The added value and benefit of sample preparation on LOC are clear, especially in the proteomics field. The perfect match between the scaling efficiencies of enzymatic reactions with the decreasing volumes provided by droplet-sized microreactors, proteomics, and MS' ability to deal with low-volume samples make it an ideal candidate for wide-spread usage within the proteomics community. However, robust datasets, are demonstrated sparsely, one example is continuous monitoring of enzyme kinetics on a micro-array plate.

We foresee chipLC–MS becoming commonplace in upcoming years, especially since several commercial systems that offer increased throughput, sensitive analysis and allow easy operation are already available. The main advantage of chipLC–MS lies in its reduced sample usage and therefore it will be predominantly used for small sample volume applications.

ChipCE–MS systems need further improvements in robustness before they can be applied on a larger scale.

Table 1

Overview of incentives and pre-requisites for the adoption of LOC-MS devices

Incentives	Examples
Small volume analysis	Single cell analysis, neonate screening, animal model fluids and tissues, all direct infusion and separation approaches, trace analysis, push pull probe, capillary gap sampler, clinical diagnostics and drug screening
High throughput, parallelization and automation	Drug screening (*in vitro* and assay-based), sample preparation in proteomics, DBS analysis, multinozzle emitter array, Advion systems, capillary gap sampler
Time-continuous monitoring	*In vitro* cell models, enzymatic assays
Molecular interaction assays	Enzyme reactions, frontal affinity chromatography, H/D exchange
On-line sample preparation	On-line proteolysis, integrated proteomic workflows
Pre-requisites	
Robust datasets	
Standardisation and protocols	
Clear added value and benefit	
Cartridge integration/'plug and play'	
Commercialisation of set-ups	

Work is currently focussed on make-up flows, which we expect to lead to more robust systems.

Lastly, we foresee increasing attention for coupling *in vitro* cell models (such as organ-on-a-chip and 3D cell culture) to MS. Pharmaceutical companies are increasingly interested to make use of such devices to gain additional information efficacy and toxicity of their compounds in the discovery and pre-clinical stage.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.copbio.2014.08.009.

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- •• of outstanding interest

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