A.1 Supplementary material and methods information

A.1.1 Protein pI markers labeling with FITC

Protein pI markers (Broad pI Kit, pH 3–10, GE HealthCare) were labeled with FITC for fluorescence monitoring of IEF experiments: FITC was dissolved in DMSO to 2.5 mg/ml final concentration. Then, the FITC-protein conjugation buffer was prepared by adding 12.5 µl of the FITC solution to 312.5 µl of 0.1 M triethylammonium bicarbonate, pH 8 (Sigma-Aldrich). This buffer was then used to solubilize 325 µg of protein pI markers. The mixture was let to rest in the dark and at room temperature (RT) for 2 h. Removing unbound fluorescent label was carried out by centrifuging the mixture through a 10 kDa MWCO filter (Amicon) at 13,500 × g for 30 min at 4 °C. Then, 500 µl of milli-Q water was added to the filter and centrifuged again using the above mentioned conditions but for 45 min. The flow through was discarded and the filtered material was re-dissolved in 650 µl milli-Q water.

A.1.2 µFFE operation proof-of-principle details

A.1.2.1 µFF-ZE of FITC, rhodamine B and pyronin Y

10 mM HEPES, 20 mM Bis-tris, pH 8 was used as separation buffer in ZE mode. The sample solution, termed “sZE” in fig. 4A, was prepared by diluting FITC, rhodamine B and pyronin Y all to a final concentration of 10 µg/ml in separation buffer. The sZE, sheath buffers and flow rates are indicated in the figure and table 1. The flow rates have been selected to ensure that the sample leaves the device through the middle outlet when no voltage was applied. The reservoirs were flushed with PBS. Voltages up to 200 V were evaluated.

A.1.2.2 µFF-FSE of FITC

The sample solution, termed “sFSE” in fig. 5A, was prepared by diluting FITC to a final concentration of 10 µg/ml in HEPES/Bis-tris buffer. The sFSE, sheath buffers and flow rates are indicated in the figure and table 1. The difference to the ZE mode is the use of an additional PBS sheath flow close to the anode. The combined flow rates of the PBS and adjacent HEPES/Bis-tris sheath flows were equilibrated to equal the sheath flow closer to the cathode. Voltages up to 200 V were evaluated.
A.1.2.3 µFF-IEF of FITC labeled protein pI markers

The sample solution, termed “sIEF” in fig. 6A, was prepared by adding 2% (v/v) carrier ampholytes (CA, pH 3-10, Sigma-Aldrich), for pH gradient generation, to the protein pI marker stock solution prepared in section [A.1.1]. The electrolyte reservoirs were filled with 20 mM phosphoric acid, 5 mM aspartic acid, pH 2.2, as anolyte, and 20 mM sodium hydroxide, 50 mM arginine, pH 12.0, as catholyte. Furthermore, in the microchannels, the sIEF was sheathed using the same solutions as those flowing in the reservoirs. Flow rates used are indicated in fig. 6A and table 1. The separation was performed at a constant voltage of 100 V.

A.1.3 Separation of protein pI markers in µFF-ZE and protein identification as a function of outlet by mass-spectrometry

Separation of a stock solution of protein pI markers was tested in ZE mode, similarly to that presented in the respective proof-of-principle (fig. 4). The flow rates used are equal to those used for the proof-of-principle. The sZE was prepared by diluting protein pI markers to a final concentration of 0.5 mg/ml. The fractionated flow was collected into a 96 well plate in two replicates of 5 min time intervals. Collected fractions were analyzed by mass spectrometry based proteomics. Briefly, proteins in each fraction were precipitated by 10-fold dilution with ice-cold ethanol and incubation at -40 °C for 2 hours. Precipitated proteins were collected by centrifugation at 18,000 × g for 30 min (4 °C). The supernatant was carefully removed and pellets were air dried for 15 min at RT. Pellets were re-dissolved in 4 M guanidinium chloride and diluted to a final concentration of 0.2 M using 50 mM ammonium bicarbonate buffer. Cysteine residues were reduced by addition of 10 mM dithiothreitol for 30 min at 56 °C and alkylated using 30 mM iodoacetamide for 30 min at room temperature in the dark. Protein concentrations per fraction were estimated based on the stock mixture concentration and taking into account (i) that proteins were present in equal amounts, (ii) the results from previous separations, and (iii) the expected migration based on pI values. Samples were digested overnight with 1:20 (protein:enzyme) trypsin (Promega Sequencing Grade) at 37 °C. Afterwards, samples were analyzed by nano LC-MS/MS on an LTQ Orbitrap XL coupled to an Ultimate 3000 Liquid Chromatography system (both from Thermo Fisher Scientific). Around 1/10 of each fraction was loaded onto a trap column (Acclaim PepMap100; C18; 100 µm × 2 cm) with 0.1% trifluoroacetic acid (TFA). Peptides were separated on the main column (PepMap100 C18; 75 µm × 15 cm), using a binary gradient ranging from 3-40% solvent B (84% acetonitrile, 0.1% formic acid) in solvent A (0.1% formic acid) for 127 min at 60 °C and a flow rate of 300 nl/min. Survey scans were acquired in the Orbitrap with a resolution of 60,000, using the polysiloxane m/z 371.1012 as lock mass. MS² scans of the ten most intense signals were acquired in the ion trap using CID fragmentation (normalized collision energy of 35, isolation width of 1.0 m/z) with a dynamic exclusion of 20 s. Raw data were processed with Proteome Discoverer 1.4 (Thermo Fisher Scientific) using Mascot v2.4.1 (Matrix Science) and a database containing the Uniprot sequences of the proteins present in the mixture with a background of 7022 proteins, including the SGD database (7031 target sequences). The following settings were applied: trypsin as enzyme allowing two missed cleavages, carbamidomethylation of cys-
teine (+57.0214 Da) residues as fixed modifications, oxidation of methionine (+15.9949 Da) as variable modification, mass tolerances for MS and MS/MS were set to 10 ppm and 0.5 Da, respectively. Peptide spectrum matches (PSM) of the target proteins that passed a medium confidence filter (5% FDR) were considered, as the search space with 7022 background proteins within the database renders a false positive PSM match to the proteins in the mix highly unlikely. The number of peptide spectrum matches (PSM) per protein and fraction was used as a direct measure of protein separation across fractions.

A.2 Earlier developments

We considered that our earlier developments would be of the interest of the reader, but in order to maintain the main paper concise and clear we decided to revert its details to the supplementary information.

A.2.1 What was different in the early development stage?

In early development stages we used a device as represented in fig. S1A. In terms of the microfluidic channel configuration, only minor modifications exist at the inlet and outlet design levels. The two main differences were the device material constitution and the fact that the electrolyte reservoir was open to the air. The device fabrication, described in detail below, included a glass slide, as a rigid support, to which a thin PDMS sheet containing the microfluidic structures was bonded. Then, a bulk PDMS structure with bonded PC membranes and electrolyte reservoirs was aligned and bonded to the microfluidic structures. The use of open reservoirs poses electrical and chemical risks (e.g., use of acid and bases in IEF) to the user. Also, the PDMS thermal conductivity is about $10 \times$ lower than that of glass and therefore a double PDMS layer microfluidic fabrication scheme offers limited heat dissipation. Limited heat dissipation may increase temperature substantially and cause bubble formation. These drawbacks were fixed in the µFFE device used in the main paper by: (i) sealing the electrolyte reservoir with a glass slide, and (ii) direct integration of the microfluidic structures with a glass substrate by bonding a PDMS slab, containing the microchannels and PC membranes, directly to glass.

A.2.1.1 Fabrication

The microfluidic containing layer (~1 mm thick) was fabricated by spin coating PDMS on a SU8 mold containing the microfluidic structures at 100 rpm during 1 min and cure at 100 °C for 15 min. Corona discharge (ETP BD20AC, USA) was applied to the PDMS while still on top of the wafer, and to a large glass slide (50 × 75 mm$^2$). The glass slide was aligned by hand on top of the PDMS, let to bond during 1 h and then removed from the wafer. A top 4 mm thick layer was fabricated by injection molding, using in-house micromachined PMMA plates which contained structures for the definition of electrolyte reservoirs, rectangular access holes to the microfluidic structures and holes for insertion of 20 G blunt needles for the definition of inlets/outlets. Membrane bonding was performed as described in the main paper. Here, the difference is that the membrane was bonded to the PDMS layer opposite to that containing the microchannels. Then, the top PDMS slab was bonded to the bottom glass/PDMS layers via O$_2$ plasma.
Figure S1: Early µFFE developments. A) Perspective view of the microfluidic device. B) Schematic microfluidic device top view: 3 inlets, 9 outlets and reservoirs situated to the side where electrodes are placed to apply 150 V. The solutions and flow rates used to perform a pH gradient are indicated. The bottom image shows the colorimetric pH scale supplied by the pH indicator solution vendor. C) Photo of the device after operation. A color gradient is observed at the outlets.

Platinum electrodes were placed on the electrolyte reservoirs and connected to a power source, similar to that described previously (section 3.2).

A.2.1.2 pH gradient for IEF: colorimetric visualization by using a universal pH indicator

A universal pH indicator was used in an experimental setup suitable for µFF-IEF, for simplicity of pH gradient visualization (fig. S1). Flow rates and solutions used are displayed at the inlets in fig. S1B. The sample solution contained 2% carrier ampholytes. All solutions contained 0.1% 2-hydroxyethyl cellulose. The basic and acid solutions used to sheath the sample were also used at the respective electrolyte reservoirs. After operation at a voltage of 150 V a clear color gradient was observed at the outlets (fig. S1C), which may be compared with the color scale provided by the pH indicator vendor in fig. S1B. A linear pH gradient was obtained.

A.2.1.3 Separation of DNA as a function of the number of base pairs

Figure S2 shows the experimental setup and results on the separation of DNA as a function of the number of base pairs. In order to separate DNA by size, we used a highly viscous solution of 0.5% HEC, 0.5% glycerol in 0.5× TAE buffer which acts as a sieve during µFF-ZE operation. A 5 fragment (50, 200, 400, 850, 1500 bp) DNA ladder intercalated with SYBR green (Thermo Scientific) was diluted in milli-Q water and injected in the microfluidic device through inlet “b”, in an asymmetric inlet configuration in order to grant larger separation range, as represented in fig. S2A. The sieve solution was injected through inlets “a” and “c”. Figure S2B shows merged fluorescence microscopy images at the separation bed’s outlet region during µFF-ZE operation. Five distinct bands, equal in number to the DNA ladder fragment library used, were collected at different outlets. However, band separation was not stable over time and significant band broadening was observed shortly after (1-2 min) formation of the bands displayed in the figure. Further work is required to ensure separation control and long-term stability, namely by optimizing the sieve solution and flow rates.
Figure S2: Separation of DNA according to the number of base pairs. A) Schematic microfluidic device top view: DNA solution injected through inlet “b” and sheathed by a highly viscous buffer which acts as a sieve. The sieve solution was injected through inlets “a” and “c”. B) Merged fluorescence microscopy images at the microfluidic’s device outlet region: a 5-fragment DNA ladder labeled with the SYBR green fluorescence intercalator was separated into bands.