species may occur, such that the difference in migration speed exactly compensates for the distance between the two excitation WGs within one DW. As a result, the two fluorescence peaks are simultaneously detected by the color-blind PMT in this DW. However, the peaks do not coincide in the other DW, because the swapping of the excitation wavelength would indeed enhance the effect of different migration speeds instead of creating a degeneracy. (ii) From the double detection, additional information can be deduced about flow speed, plug broadening, etc. (iii) Low-concentrated species that disappear under the background noise owing to plug broadening while migrating to DW2, will still give rise to weak fluorescence peaks at DW1, thus providing extra information that would otherwise be lost.

In principle, such a set-up can be operated with a single PMT, e.g., by collecting the fluorescence from the two DWs by two fibers glued to the top of the chip [11] or two 3D-integrated WGs [16], and transporting the signals to the same PMT, thereby simplifying the whole setup and reducing its size. In this case, to achieve an unambiguous detection, it is a critical prerequisite to choose a sufficiently large distance between the two DWs, such that the first peak in DW2 appears only after the last peak in DW1 has disappeared. Since our experimental setup requires detection by a PMT through a microscope, only one of the two DWs can be monitored at a time. Hence, it is currently not possible in our setup to monitor both DWs simultaneously.

Therefore, to demonstrate the potential of dual-point, dual-wavelength monitoring for internal size calibration, we present a simulation of a MCE experiment based on the logarithmic relationship between DNA size and migration speed, as well as the square-root dependence of plug broadening on the DNA size, as established in an earlier experimental analysis [15]. The Poissonian dependence of the electropherogram peak width on the DNA size is represented in Fig. 3 as the evidently broader peaks in DW2 in comparison with DW1, with the broadening being related to the DNA size by a square-root dependence, while the migration times of the DNA molecules have been simulated based on their estimated migraton speeds, which depend on their sizes. In this simulation, we consider the separation of a (usually unknown) DNA sample under investigation, consisting of green-labeled molecules S1 (250 bp), S2 (300 bp), S3 (450 bp), and S4 (700 bp), flown against a (known) DNA reference consisting of red-labeled molecules R1 (150 bp), R2 (355 bp), and R3 (1000 bp).

Figure 3 (top, black curve) shows a simulated electropherogram that would be generated if the fluorescence is detected by a color-blind PMT simultaneously from both DWs as a result of laser excitation (with a mutually swapped order of excitation wavelengths) through the four WGs. The distance between the two DWs and/or the minimum and maximum sizes of DNA molecules are chosen such that all DNA molecules migrate first through DW1 and then through DW2, i.e., the luminescence signals occurring from the two DWs are separated in time, as indicated by the dashed line.

The deconvolution of the detected signal into the individual signals obtained from the sample and reference DNA molecules is presented in Fig. 3 (center, red curve, and bottom, green curve). The sizes of the reference molecules are chosen such that the smallest and largest base-pair sizes belong to reference molecules, hence they can be easily identified. Based on the *a priori* known sizes of the reference molecules, one can make use of the known dependence of the relative migration time on DNA size [15] to identify the third, medium-sized reference molecule in both windows, because its peak principally shifts from DW1 to DW2 in the same manner with respect to the peaks of the unknown molecules as the first and last reference molecule, and calibrate the remaining peaks of the electropherogram for varying environmental conditions, i.e., to accurately determine the sizes of the corresponding sample molecules based on their migration times relative to the migration times of the known reference molecules. The third, medium-sized reference molecule provides an indication of the experimental deviation from the expected migration due to parameter drifts. While the R2 and S3 peaks incidentally overlap in DW1 owing to their unfortunate size difference, they are well resolved as R'2 and S'3 in DW2 as a result of swapping the excitation wavelengths. In such a setting, it is straight forward to identify each peak in both parts of the diagram, DW1

and DW2, thus enabling unambiguous analysis of two sets of exclusively color-labeled DNA molecules of different origin by detection with a single color-blind PMT.
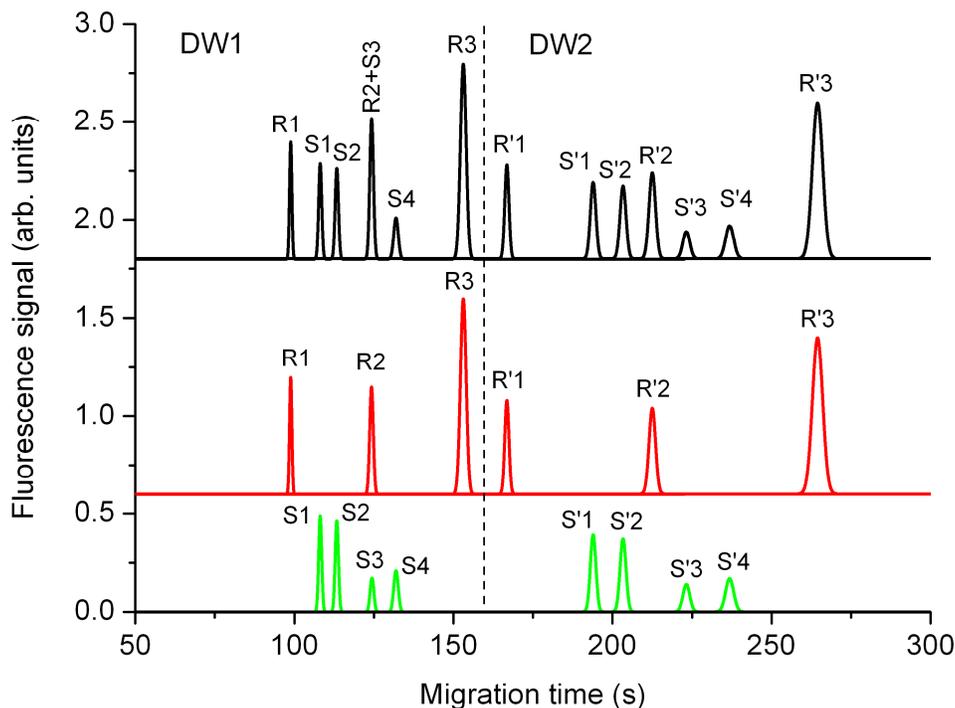

Fig. 3. Simulated electropherograms as would be detected from the two DWs with swapped excitation wavelengths during an experiment with internal calibration using a green-labeled DNA sample ("S") consisting of four different molecule sizes (250 bp, 300 bp, 450 bp, and 700 bp) and a red-labeled DNA reference ("R") consisting of three different molecule sizes (150 bp, 355 bp, and 1000 bp). Fluorescence signals S were excited through WG1, R through WG2, R' through WG3 and S' through WG4.

5. Conclusions

We have analyzed the electrophoretic separation of fluorescently labeled DNA molecules along a microfluidic channel with integrated waveguides in a dual-point, dual-wavelength setting. Using a single detection window, we demonstrate highly accurate detection of single-nucleotide insertion/deletion, as is relevant in genetic diagnostics – detection of anomalies in genetic samples obtained from a patient with respect to their healthy genetic counterparts. Furthermore, based on earlier experimental DNA separation data, we present a simulation of a separation experiment in a setup that uses two detection windows, with the two excitation wavelengths swapped in the second one, for internal calibration/referencing of DNA sizes. This can be a highly effective approach for intrinsically eliminating the undesirable effects of several flow parameters on calibration of a MCE separation system by flowing a well-known reference sample simultaneously with the unknown sample. The results presented in this paper bear the potential of leading to a new generation of compact optofluidic devices for use in point-of-care diagnostics.

Acknowledgements