Finding the needle in the haystack - detection of circulating endothelial cells with a new EpiSpot

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Revascularisation in the setting of critical limb ischema (CLI) is the treatment objective. For the evaluation of an angiogenic effect, peripheral circulating endothelial cells (CECs) and endothelial progenitor cells in the blood stream should be detected in an EpiSpot assay system. The development of the assay and proof of principle with human umbilical vein endothelial cells (HUVEC) are part of this work.

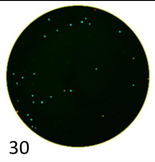
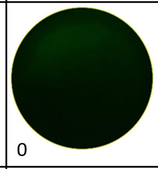
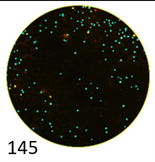
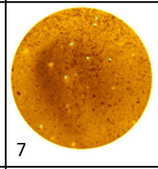
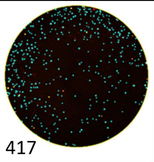
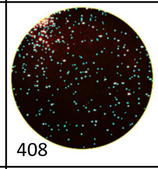
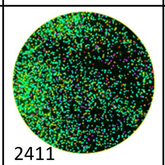
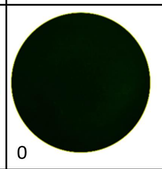
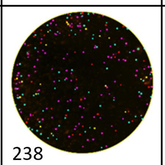
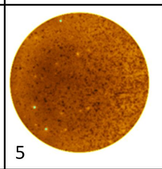
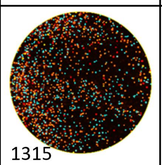
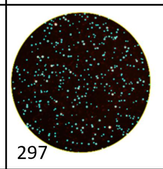
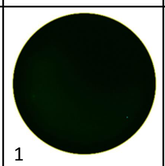
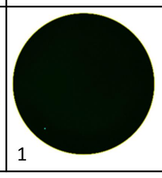
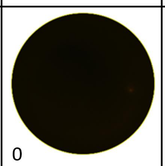
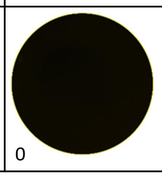
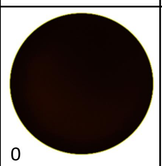
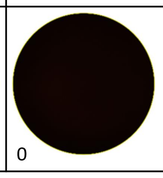
24 h	E-Selectin		vWF		IL-6			
	HUVECs	PBMCs	HUVECs	PBMCs	HUVECs	PBMCs	HUVECs	PBMCs
NC	 30	 0	 145	 7	 417	 408	E-Sel/vWF: 0 vWF/IL-6: 9 E-Sel/IL-6: 6 triple: 1	E-Sel/vWF: 0 vWF/IL-6: 0 E-Sel/IL-6: 0 triple: 0
TNFα + Vimentin	 2411	 0	 238	 5	 1315	 297	E-Sel/vWF: 185 vWF/IL-6: 40 E-Sel/IL-6: 604 triple: 78	E-Sel/vWF: 0 vWF/IL-6: 0 E-Sel/IL-6: 0 triple: 0
without cells and stimulant	 1	 1	 0	 0	 0	 0	E-Sel/vWF: 0 vWF/IL-6: 0 E-Sel/IL-6: 0 triple: 0	E-Sel/vWF: 0 vWF/IL-6: 0 E-Sel/IL-6: 0 triple: 0

Fig 1 24 h incubation of PBMCs and HUVECs with TNFα+Vimentin as stimulating factor and the antibodies against each other without cells and without stimulant. The aim is to identify endothelial cells by simultane secretion of two or more analytes. For E-Selectin only HUVECs show spots (left), which number increases after stimulation. The vWF can be detected in HUVECs (middle) and is induced through TNF α +Vimentin, which is documented for IL-6 (right) as well. The PBMCs show high background for vWF and spots for IL-6. Before stimulation the spots are nearly all single positive. After stimulation double and triple positive spots for HUVECs are detected. In conclusion, endothelial cells can be disciminated against PBMCs.

Therefore, vascular endothelial growth factor (VEGF), the coagulation factor VIII (FVIII), E-Selectin, von Willebrand Faktor (vWF) and Interleukin-6 (IL-6) antibody pairs were tested, and concentrations and incubation times were evaluated with HUVECs in combination with PBMCs (Fig. 1). Flow-cytometric analysis was done in parallel to determine the applicability of HUVECs as a model for circulating endothelial cells. Beside this, isolated and enriched endothelial cells (ECs) from peripheral blood were characterised as CECs by using flow cytometry (Fig. 2).

E-Selectin has been selected to identify ECs unambiguously in the established assay system. This was proven by titration of HUVEC in PBMCs, and recovery rates of 10 HUVEC / 200.000 PBMCs were achieved (Fig. 3).

A

Tube Name: OF
Sample ID:
Volume(µL): 24,5

Population	Events	% Total	% Parent	Events/µL(V)
All Events	100000	100,00%	100,00%	4075,51
debris	63666	63,67%	63,67%	2594,71
lymphocytes	34469	34,47%	56,20%	1404,79
singlets	61335	61,34%	96,34%	2499,71
viable	61117	61,12%	99,64%	2490,83
CD34+	0	0,00%	0,00%	0,00
Q1-UL	0	0,00%	####	0,00
Q2-UL	0	0,00%	####	0,00
CD133+	2	0,00%	0,00%	0,08
146+	99	0,10%	0,16%	4,03

B

Tube Name: cEC
Sample ID:
Volume(µL): 297,2

Population	Events	% Total	% Parent	Events/µL(V)
All Events	76565	100,00%	100,00%	257,62
debris	13488	17,62%	17,62%	45,38
lymphocytes	5471	7,15%	44,72%	18,41
singlets	12234	15,98%	90,70%	41,16
viable	10011	13,08%	81,83%	33,68
CD34+	2533	3,31%	25,30%	8,52
Q1-UL	30	0,04%	1,18%	0,10
Q2-UL	1870	2,44%	73,83%	6,29
CD133+	1969	2,57%	19,67%	6,63
146+	73	0,10%	0,73%	0,25

Fig 2 FACS analysis of CEC enrichment experiments. A, original fraction, where almost no ECs or CECs can be detected. After enrichment, the fraction of ECs identified as CECs clearly increases (B), but still these cells are hard to find in the periphery of a healthy donor.

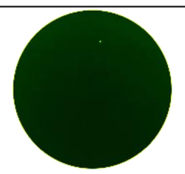
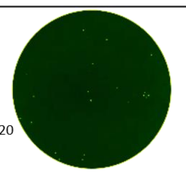
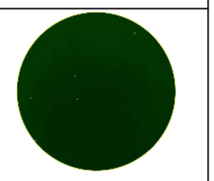
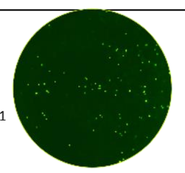
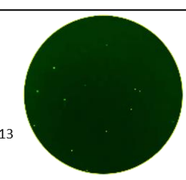
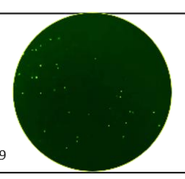
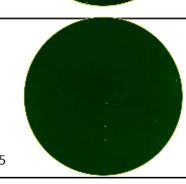
Number of HUVEC/ Well	E-Selectin	Number of HUVEC/ Well	E-Selectin	Number of HUVEC/ Well	E-Selectin
Control PBMCs	 1	100	 20	5	 6
500	 91	50	 13	1	 3
200	 39	10	 5		

Fig 3 Titration of HUVECs spiked to a certain PBMC fraction. Nmber of PBMSc were constantly 200.000 per well, whereas number of spiked HUVEC changed from 500 / well down to calculated 1 cell/ well.

The developed assay-system can detect low numbers of endothelial cells in general. The amount of circulating endothelial cells in healthy volunteers is relatively low, therefore additional experiments will reveal the usefulness of the assay for CLI treatments and will allow to define the sensitivity in patient samples.

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