Multiplex Electrochemiluminescence Immunoassays for Phenotyping of Intact Extracellular Vesicles (EVs)

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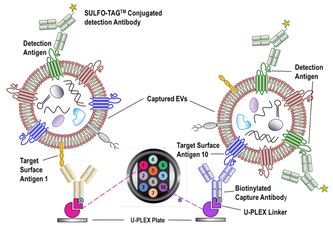
Introduction

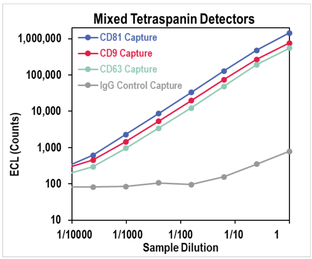
There is a need for better techniques for characterizing EV populations. We developed a sensitive multiplexed electrochemiluminescence (ECL)-based assay format to characterize EVs in cell-conditioned medium (CCM) and human biofluids. Here we use the assay format to analyze EV samples for the presence of 66 EV surface proteins, and to identify changes in EV phenotype associated with different cell lines, purification methods and growth conditions.

Methods

Multiplexed EV Immunoassays

Biotinylated capture antibodies are each coupled to one of ten unique U-PLEX® Linkers, which self-assemble onto unique array elements (or "spots") on the U-PLEX plate. EVs presenting target surface antigens recognized by the capture antibodies bind to the associated spots. Bound EVs are detected using detection antibodies conjugated with electrochemiluminescent labels that recognize detection antigen(s) on the EVs. MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® microplates.





Tetraspanin Assays for Total EVs

U-PLEX arrays of capture antibodies targeting tetraspanin proteins CD9, CD63, CD81 and a negative control antibody are paired with a cocktail of detection antibodies for all three tetraspanin targets. Previously we showed these multiplexed arrays performed the same as the individual capture antibodies. Purified EVs from a known cell line are used as a calibrator to normalize signals between plates and experiments. The signals produced by these assays are proportional to the total EV concentration in the samples, and the control antibody is used to assess non-specific binding of EVs to the plate and ensure reliability of the data.

Cell Conditioned Media Samples

PC-3 (ATCC® CRL-1435™) and HCT-116 (ATCC® CCL-247™) cell lines were cultured at MSD using complete medium and at ATCC using EV-depleted medium. An hTERT-immortalized mesenchymal stem cell line (ATCC® SCRC-4000™) was cultured at ATCC in serum-free medium. Primary mesenchymal cells were cultured at Creative Biolabs in serum-free medium. Primary preadipocytes from subcutaneous adipose tissue and omental adipose tissue were grown at ZenBio in serum-free medium. EVs were measured in the raw conditioned media and in purified EV fractions prepared as described in the following section.

Sample Purification

Conditioned Medium from cell lines grown at ATCC were subjected to tangential flow filtration (TFF) at ATCC to purify and concentrate EVs. These materials are now available as products from ATCC: ATCC® CRL-1435-EXM™, ATCC® CCL-247-EXM™, and ATCC® SCRC-4000-EXM™. Conditioned medium from each of the sources were subjected to several purification methods at MSD: ultrafiltration by centrifugal filter units, precipitation by a volume exclusion reagent, size-exclusion chromatography using sepharose CL-2B. Primary preadipocyte CCM was also purified by ultracentrifugation at ZenBio.

Results

Comparing Total EV in each cell conditioned medium and EV isolate

Multiplex tetraspanin EV assays were used to assess the total EV content of each CCM sample and EV isolate. Signals from each EV isolate were divided by the signal in the most concentrated sample (HCT116 TFF) to produce a an relative EV concentration, compared to that sample. These estimated concentrations were only weakly dependent on which tetraspanin capture antibody was used. We used the average of the three markers to adjust data for overall EV concentration in the following tables. SEC produced much lower concentrations than the other methods as it diluted rather than concentrated the samples.

Relative Concentration of total EVs in each sample

Cell Type	Source	Purification	CD63 Capture	CD81 Capture	CD9 Capture	TSPAN Average
HCT116	ATCC	None	0.053	0.052	0.054	0.053
HCT116	ATCC	UF	0.059	0.058	0.059	0.059
HCT116	ATCC	PEG	0.041	0.038	0.041	0.040
HCT116	ATCC	SEC	0.0028	0.0036	0.0050	0.0038
HCT116	ATCC	TFF	1.0	1.0	1.0	1.0
HCT116	MSD	None	0.12	0.15	0.15	0.14
HCT116	MSD	UF	0.12	0.15	0.14	0.14
HCT116	MSD	PEG	0.083	0.10	0.10	0.096
HCT116	MSD	SEC	0.0071	0.012	0.017	0.012
PC3	ATCC	None	0.14	0.097	0.10	0.11
PC3	ATCC	UF	0.16	0.12	0.12	0.13
PC3	ATCC	PEG	0.108	0.085	0.095	0.096
PC3	ATCC	SEC	0.020	0.020	0.031	0.024
PC3	ATCC	TFF	0.50	0.38	0.40	0.43
PC3	MSD	None	0.15	0.094	0.10	0.11
PC3	MSD	UF	0.14	0.096	0.096	0.11
PC3	MSD	PEG	0.086	0.065	0.065	0.072
PC3	MSD	SEC	0.019	0.020	0.027	0.022
MSC - tert immortalized	ATCC	None	0.086	0.060	0.045	0.064
MSC - tert immortalized	ATCC	UF	0.078	0.057	0.043	0.059
MSC - tert immortalized	ATCC	PEG	0.044	0.034	0.026	0.035
MSC - tert immortalized	ATCC	SEC	0.0078	0.0064	0.0057	0.0067
MSC - tert immortalized	ATCC	TFF	0.39	0.31	0.24	0.31
MSC - Primary, adipose-derived	Creative Biolabs	None	0.015	0.0045	0.0023	0.0073
MSC - Primary, adipose-derived	Creative Biolabs	UF	0.011	0.0040	0.0022	0.0059
MSC - Primary, adipose-derived	Creative Biolabs	PEG_Precip	0.0075	0.0037	0.0022	0.0045
Preadipocyte - Primary, omental	ZenBio	None	0.028	0.012	0.010	0.016
Preadipocyte - Primary, omental	ZenBio	UF	0.022	0.010	0.009	0.014
Preadipocyte - Primary, omental	ZenBio	PEG_Precip	0.014	0.0082	0.0075	0.010
Preadipocyte - Primary, omental	ZenBio	SEC	0.0020	0.00058	0.00059	0.0010
Preadipocyte - Primary, omental	ZenBio	None	0.025	0.012	0.0066	0.014
Preadipocyte - Primary, omental	ZenBio	UF	0.012	0.0053	0.0032	0.0070
Preadipocyte - Primary, omental	ZenBio	PEG_Precip	0.0052	0.0043	0.0027	0.0041
Preadipocyte - Primary, omental	ZenBio	SEC	0.0022	0.00061	0.00055	0.0011
MSC - Primary, adipose-derived	ZenBio	UC	0.36	0.39	0.20	0.82

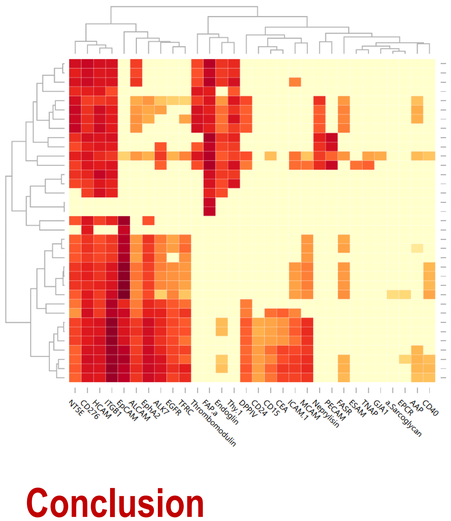
Assessing EV Phenotypes by Multiplex Surface Marker Profiling

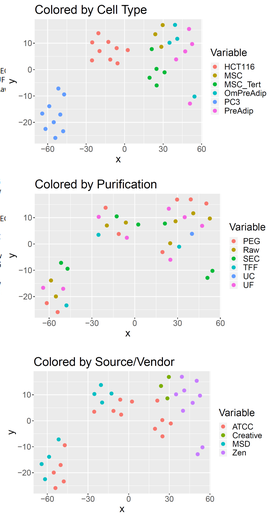
All samples listed above were assayed for EVs with sixty-six surface markers including the three tetraspanins discussed above. The assays were divided into 10-plex panels, each panel including capture antibodies for 9 surface markers and a negative control antibody. A mixture of labeled antibodies for the three tetraspanins was used for detection. Signals for each marker were expressed as a percentage of the average of the CD9, CD63 and CD81 capture signals for that sample, approximating the percentage of the EVs in the sample expressing this marker. Values were omitted from the table where the specific capture produced less than 3 times the signal produced by the control antibody. EVs with thirty of the surface markers were detectable in at least one of the samples. Phenotypes were remarkably consistent across various samples from each cell line.

			CD63	CD81	CD9	AAP	ALCAM	ALK7	a-Sarcoglycan	CD15	CD24	CD276	CD40	CEA	DPPIV	EGFR	Endoglin	EpCAM	EPCR	EpHA2	ESAM	FAP-a	FASR	GJA1	HGAM	ICAM-1	ITGB1	MCAM	Meprrylin	NTSE	PECAM	TFRC	Thrombomodulin	Thy-1	TNAP
HCT116	ATCC	Neat	86	119	95		0.87	1.2				6.5				0.58		36		5.1			0.51		4.0		5.3	0.71		2.7		2.0			
		Centrifugal UF	87	119	95		0.73	1.5				6.2				0.48		36		6.0			0.43		3.2		5.4	0.69		2.4		1.6			
		PEG_Precip	88	116	96		0.59	1.3				5.4						42		5.7					3.9		5.6	0.70		2.7		0.93			
		SEC	62	115	123							10.7						23																	
		TFF	86	120	94		1.0	0.13	0.080			10.9	0.45			1.2		95	0.10	3.5			0.90		5.2	0.54	20	0.97		2.6		0.18			
	MSD	Neat	74	126	100		1.6	1.0				9.1	0.29			0.82		73		4.4			0.65		6.1	0.61	12	1.3		6.7		0.72			
		Centrifugal UF	73	128	98		1.6	1.3				8.8	0.27			0.95		70		4.6			0.67		6.4	0.69	13	1.3		5.7		0.68			
		PEG_Precip	73	126	101		1.1	1.1				7.2	0.26			0.93		81		4.7			0.69		8.4	0.50	13	1.3		5.9		0.48			
		SEC	50	122	128							13.2						52		3.0					4.6		8.4			2.8					
PC3	ATCC	Neat	110	104	86	0.27	7.6	14.2		2.14	0.57	14.5	0.26	5.4	2.8	6.7	0.20	30.6		20			0.32		13	5.3	59	7.5		2.7		9.3			
		Centrifugal UF	105	109	86	0.20	5.8	12.7		1.61	0.49	12.5	0.20	4.1	2.4	4.4		29.4		19			0.25		11	4.2	55	5.7		2.2		7.1			
		PEG_Precip	98	108	94	0.27	4.9	8.3		1.81	0.57	13.8		4.2	2.1	4.3		32.0		19					14	3.7	55	5.3		2.3		4.3			
		SEC	72	103	125		1.9	8.8		1.73		16.5		2.3	1.4	3.2		20.6		9.2					5.3	1.1	34			0.8		5.7			
		TFF	103	108	90	0.29	6.1	13.8		1.08	0.56	17.0	0.22	3.2	2.2	4.3	0.15	42.5	0.11	19.7		0.31			14	4.8	54	6.8		2.4		2.3			
	MSD	Neat	115	101	83		6.4	7.8		0.49	0.32	11.3		0.83	2.3	3.2	0.24	13.1		16.7					12	2.5	68	7.3		4.4		3.0			
		Centrifugal UF	111	106	82		5.3	8.0		0.47	0.40	10.4		0.72	2.6	3.8	0.23	12.9		17.9					13	2.3	68	6.8		4.3		2.6			
		PEG_Precip	104	110	86		4.2	6.4		0.53	0.45	10.3		0.67	2.3	3.7		14.5		15.8					16	1.7	65	6.2		4.6		1.7			
		SEC	74	112	114		1.4	5.2				11.3			1.4	2.7		7.8		8.0					5.3		39			1.6		1.8			
MSC, tert immortalized	ATCC	Neat	118	115	67	0.34	0.83					7.0			2.6		1.7			0.38			12	1.2	17		10		2.6	20		0.49	15	14.3
		Centrifugal UF	114	118	68	0.38	0.67	0.54				6.4			2.4		1.9			0.43			9.0	1.2	18		9.5		2.4	23			14	6.2	
		PEG_Precip	111	118	71		0.83					7.3			2.7		1.9						9.0	1.4	20		10		2.7	25			16	4.9	
		SEC	102	117	81							9.1											9.4		11		3.4			7.5			11	2.7	
		TFF	108	119	73	0.21	0.39	0.18				4.7			3.6	0.13	0.71			0.74		13	0.68		5.4		6.6		6.4	16		0.11	4.9	12	
MSC, Primary	Creative Biolabs	Neat	189	80	31							5.2					3.5						14		11		8.5			4.1				14	
		Centrifugal UF	176	87	37							4.7					3.5						8.4		15		8.5								
		PEG_Precip	150	102	47							8.2					5.3						17		25		14			6.4				5.3	
Preadipocyte - Primary, Omental	ZenBio	Neat	152	91	57		5.8					20					6.2						34		13	1.2	13			14			3.7	15	
		Centrifugal UF	145	92	63		3.8					18					5.2						22		11		13			9.2			2.9	6.9	
		PEG_Precip	123	104	74		4.7					23					6.2						33		15		15			16			3.9	7.5	
		SEC	177	70	53																		26												
Preadipocyte - Primary	ZenBio	Neat	155	100	44			2.3				13			1.4		5.1				1.72		40		9.8	1.9	12	1.73	7.3	4.7	22		3.4	16	2.3
		Centrifugal UF	161	96	44			2.8				8.8					4.1						27		11		9.5		6.5	3.6	17		2.7	5.0	
		PEG_Precip	111	128	61							15					5.9						41		12		15		9.0	6.8	22			8.3	
		SEC	186	69	45																		21												
		UC	169	94	37	0.24	0.69	5.2		0.22		14	0.18		3.1	0.30	2.7	0.12		0.32			36	0.77	0.39	7.5	1.7	5.5	0.18	5.1	9.0	2.2	1.2	13	4.3	0.48

Unsupervised Clustering Analyses

Surface marker profile data from the preceding table were analyzed using two unsupervised clustering analyses. Hierarchical clustering, performed on log-transformed data using the complete linkage method, showed that EVs from the same cell line tended to show the same surface marker phenotypes independent of purification method or source. EVs from immortalized MSCs, primary MSCs and preadipocytes all had similar surface phenotypes and clustered together, except for the SEC method which produced low EV concentrations often near or below the assay detection limits. A second clustering approach, t-distributed stochastic neighbor embedding (t-SNE), carried out on non-transformed data was used to reduce the dimensionality of the data and allow the samples to be plotted in a 2-D space that captures the relationships between neighboring points. As seen with hierarchical clustering, the plots generated using t-SNE also showed the clustering of sample by cell line and type. The results demonstrated that our assay format can provide phenotypical information that is independent of EV purification method.





Conclusion

We developed multiplex EV surface marker assays and demonstrated their use for multimarker EV phenotyping. This flexible format enables rapid assay development for new EV subpopulations with or without sample purification. These results also demonstrate EV surface marker phenotyping via multiplex ECL assays may be used to distinguish EV populations from various cell types, and characterize bias introduced by purification.

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