Multiplexed Analysis of Neural Cytokine Signaling by A Novel Neural Cell-Cell Interaction Microchip

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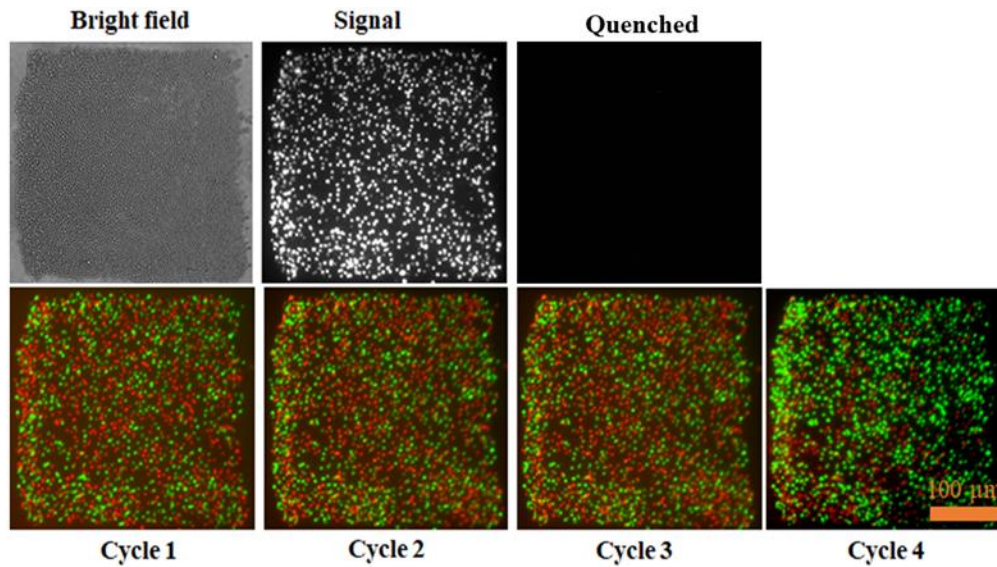[#]Corresponding authors. E-mail address: jpaluh@sunypoly.edu; Jun.wang.5@stonybrook.edu

Figure S1. Images of a MIST array for protein detection and decoding. The top panel includes a bright-field image to show all the microbeads, a protein detection image when a recombinant protein is detected, and a quenched image after 0.5 NaOH is applied to dissociate double stranded DNA. The bottom panel images are decoding images from cycle 1 to cycle 4.
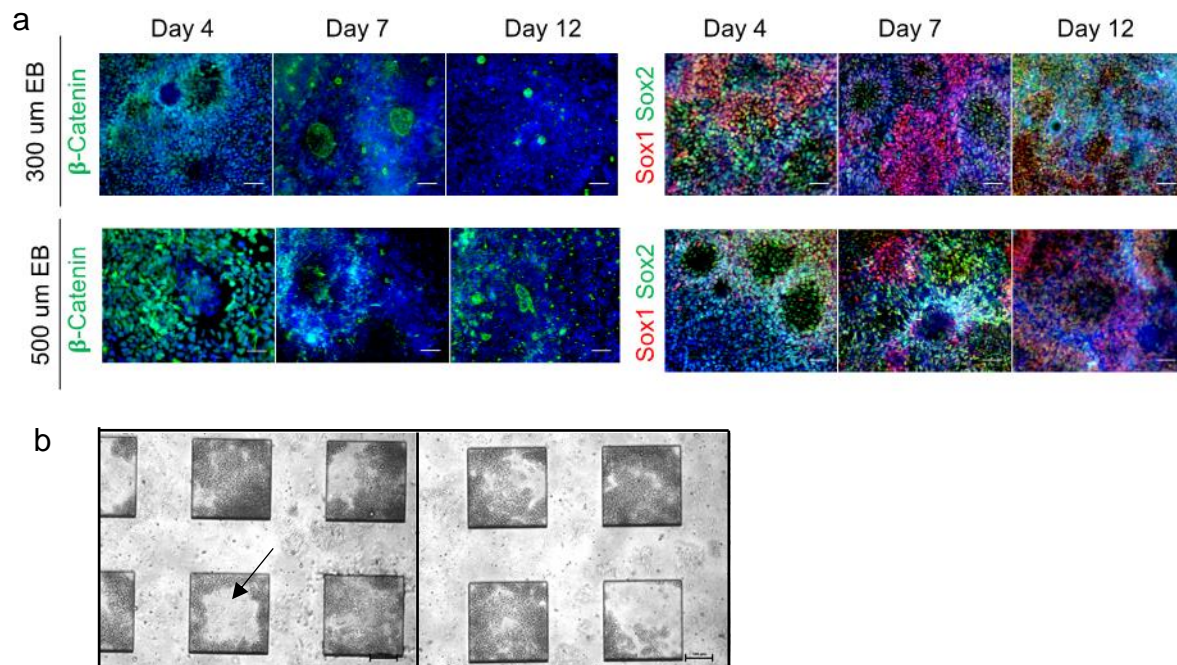

Figure S2. a) Evaluation of rosette formation on the dish from two EB sizes: 300 um and 500 um. Expression of tight junction protein B-catenin and early neural stem cell biomarkers Sox1/Sox2 were detected on days 4, 7 and 12 to determine the optimum size of PFMA chambers accommodating EBs. Scale bar is 50 um. b) Shallow chambers (100 um) did not support attachment of EBs and they were lifted off after a day.
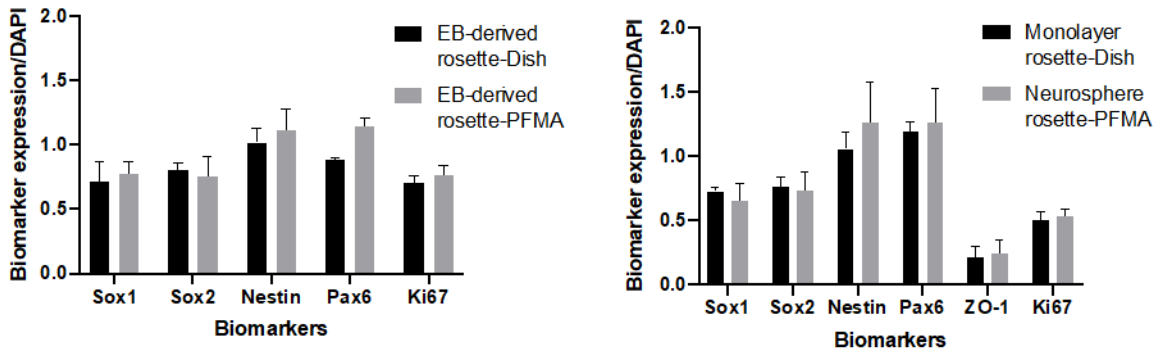
Figure S3. Expression of detected biomarkers by immunocytochemistry on three randomly selected fields from each condition was quantified using ImageJ software to quantitatively validate generated rosettes on PFMA versus dish. For statistical analysis, Two-way ANOVA and Sidak's multiple comparison test were used and no significant difference between each biomarker on the dish versus PFMA in monolayer and EB-derived rosettes was observed.
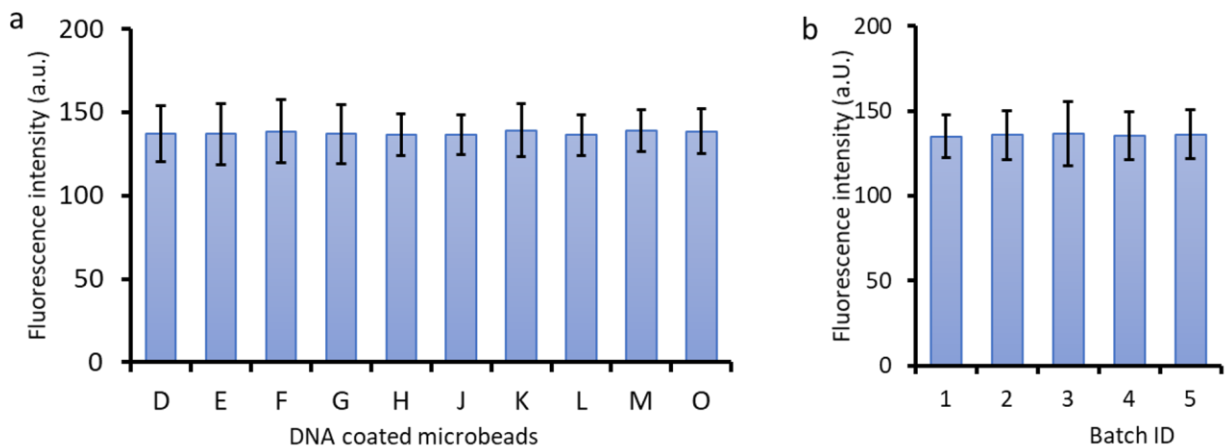

Figure S4. Reproducibility of the DNA microarray. (a) Fluorescence intensities of 10 types of DNA coated microarrays. Complementary DNAs tagged with Cy3 fluorophore are used to label the DNA microarrays, and the fluorescence intensities of 20 microbeads of each microarray are quantified and averaged. <5% variation is observed across different DNA microarrays. (b) Batch-to-batch variation of DNA microarray. D DNA was used to make 5 batches of DNA coated microbeads and microarrays. Cy3-complementary DNA was applied to show the fluorescence which was further quantified.
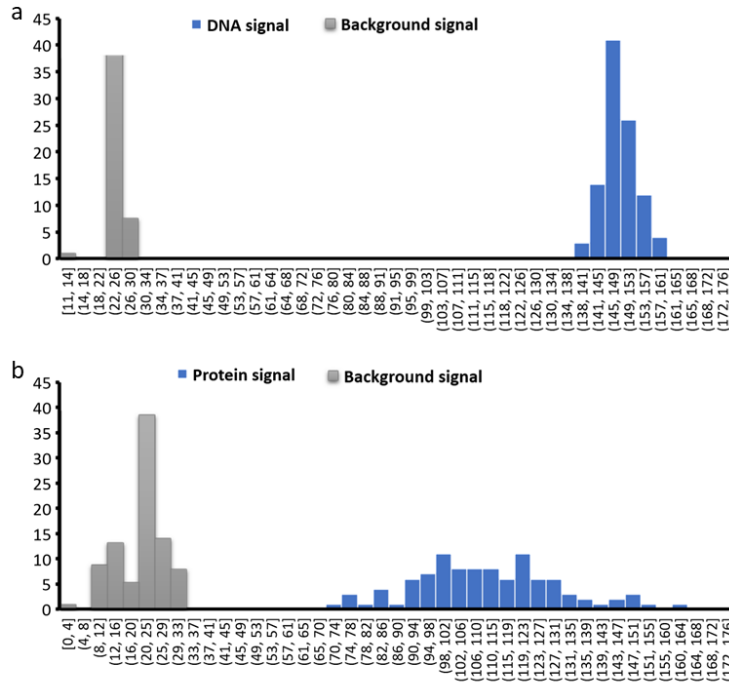
Figure S5. (a) Histograms of background fluorescence intensity and hybridized Cy3-cDNA fluorescence intensity from 100 microbeads. Background fluorescence intensity is obtained on the microbeads that are not labeled with Cy3-cDNA on the same microarray. Both histograms are widely separated without interferences. The fluorescence intensities of different Cy3-cDNA microbeads are varied by 3%. (b) Histograms of background fluorescence intensity and ELISA signal from 100 microbeads. Recombinant protein BDNF was used to measure the assay variation. The ELISA protein detection signals across microbeads are varied by 17%.
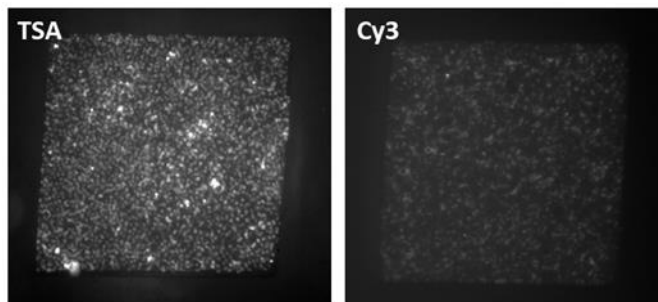
Figure S6. Image of TSA signal amplification and without amplification (Cy3) for protein detection. The average intensity of microbeads on the TSA image is only 6. 8 times higher than that on the Cy3 image.
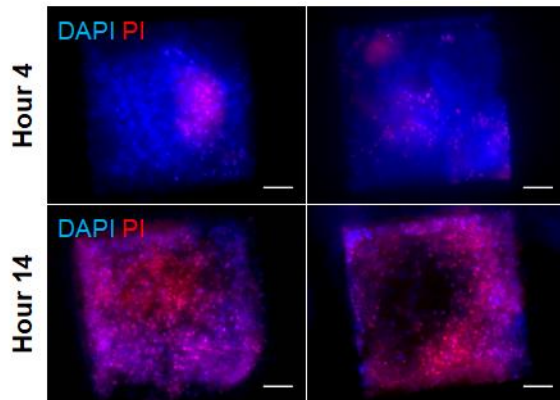
Figure S7. **Two flanking timepoints for cell viability in the NCCIM**. DAPI and Propidium Iodide (PI) reveal that cells clamped for 14 hours show considerable cell death that is not observed from an earlier 4 hour (shown) and 8 hour timepoints discussed in the text. The shorter time period did not give sufficient cytokine detection.
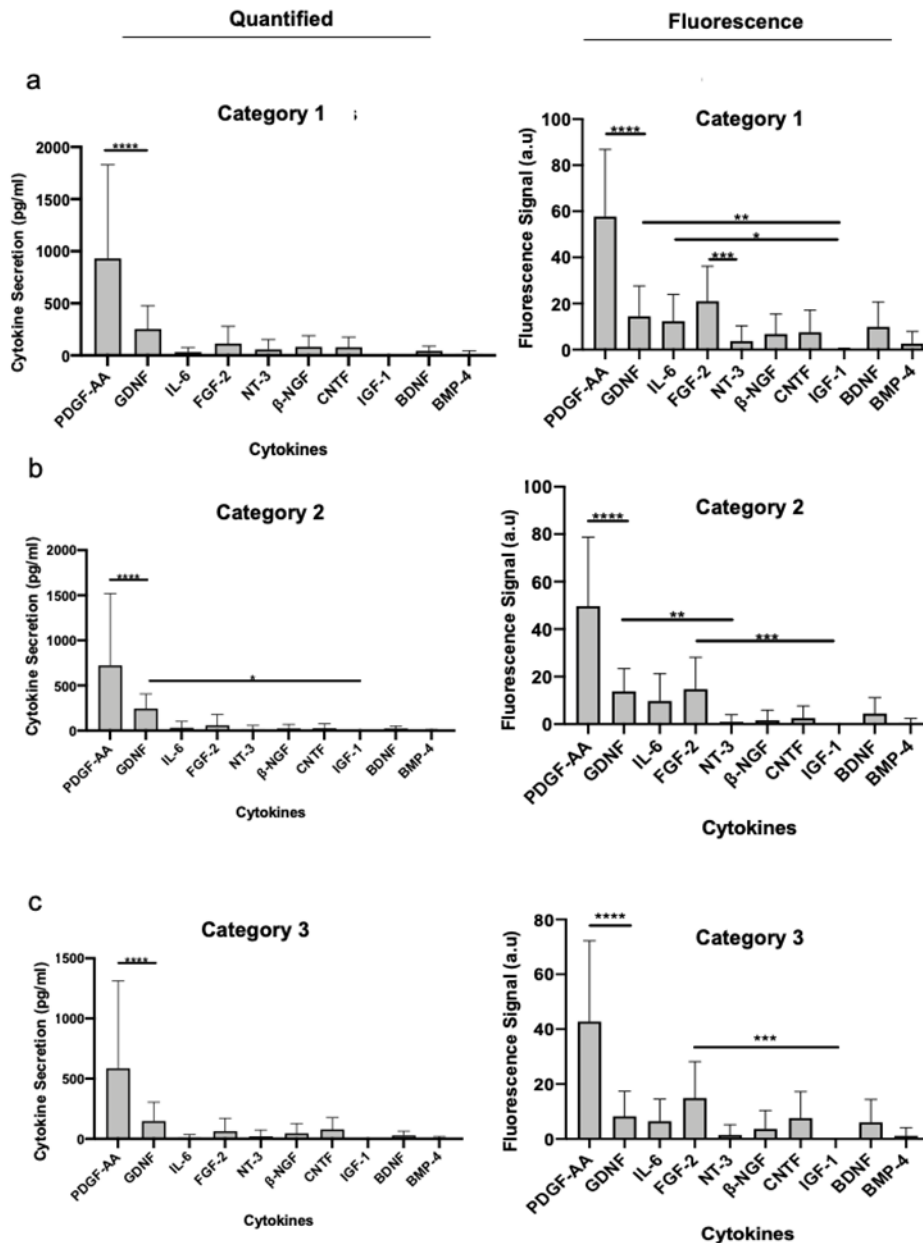
Figure S8. Plots of quantified protein concentrations and fluorescence intensity of three categories of EB-derived rosettes. Significant difference of mean values between cytokines in each category are calculated and shown as: P values: *, P<0.05; **, P<0.01; ***, P<0.01; ****, P<0.0001.
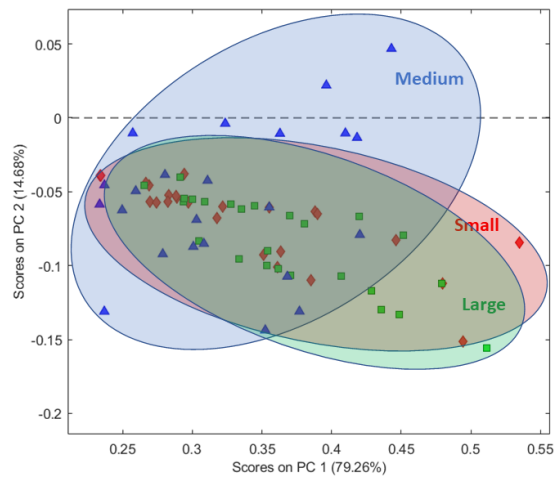
Figure S9. Principal component analysis (PCA) on NCCIM assay results of 3 sizes of rosettes. The medium size data are distinctively different while the small and large size data have significant overlap.

Table S1. NCCIM Cytokine Panel

Cytokines	Role	Signaling Pathways	References
PDGF-AA	NPC survival and proliferation	PI3K/Akt, PLC-γ, MAPK	[48, 49]
GDNF	Survival, maintenance of immature neurons, differentiation	MAPK, PI3K/Akt, PLC-γ	[50, 51]
BDNF	Survival, maintenance and differentiation of developing neurons	MAPK, PI3K/Akt, PLC-γ, GTP-ases	[62, 60]
IL-6	Neurogenesis, gliogenesis, inflammatory response	JAK/STAT, MAPK, PI3K/Akt, CREB	[52, 54]
β-NGF	Glial differentiation, inhibition of neural induction, neuronal survival	PI3K/Akt, MAPK, PLC-γ, NFKB	[63, 64]
BMP-4	Glial differentiation, inhibition of neural induction	SMAD1, 5, 8, 4	[38, 65]
NT-3	Glial survival and differentiation, neuronal survival	PI3K/Akt, MAPK, PLC-γ, NFKB	[63, 66]
CNTF	Astrocyte differentiation, glial and neuronal survival	JAK/STAT, NFKB	[63, 67]
FGF-2	Maintenance and expansion of NSC	PI3K/Akt, MAPK	[28, 57, 58]
IGF-1	Proliferation of NPC, neuronal differentiation	MAPK, PI3K/Akt	[68, 69]

Table S2. Color scheme for decoding to determine the type of protein detected by the MIST sensor.

Antibody	DNA sequence	Cycle 1	Cycle 2	Cycle 3	Cycle 4
GDNF	AAAAAAAAAAAAATACTCTGACATCTCGACCAT	Cy3	Cy3	Cy3	Cy3
PDGFAA	AAAAAAAAAAAAATAGATACTGCCACTTCACAT	Cy3	Cy3	Cy3	Cy5
IL6	AAAAAAAAAAAAATACCGTGAACCTTACCTGAT	Cy3	Cy3	Cy5	Cy5
CNTF	AAAAAAAAAAAAAATGCTCGGGAAGGCTACTC	Cy3	Cy5	Cy5	Cy5
IGF	AAAAAAAAAAAAACGCACCGCAGTTTGGTCAAT	Cy5	Cy5	Cy5	Cy5
BDNF	AAAAAAAAAAAAACCTGCTCGACAACTAGAAGA	Cy5	Cy5	Cy5	Cy3
FGF	AAAAAAAAAAAAACCGCGACCAGAATTAGATTA	Cy5	Cy5	Cy3	Cy3
NT-3	AAAAAAAAAAAAAGCCGAAGCAGACTTAATCAC	Cy5	Cy3	Cy3	Cy3
BMP-4	AAAAAAAAAAAAAACAGGTTCAGAATCCTCGAC	Cy5	Cy3	Cy5	Cy3
β-NGF	AAAAAAAAAAAAAGTCACAGACTAGCCACGAAG	Cy3	Cy5	Cy3	Cy5

Table S3. Percent Viability in NCCIM microchambers of EB-derived and neurosphere rosettes after 8 hours in the NCCIM.

Viable Cells %	EB-derived rosette				Rosette neurospheres	
	Hour 0		Hour 8		Hour 0	Hour 8
	PFMA 1 N=30	PFMA 2 N=30	PFMA 1 N=30	PFMA 2 N=30	PFMA 1 N=30	PFMA 1 N=30
80-100%	73	94	55	69	73	55
60-80%	18	6	24	25	27	23
40-60%	9	0	9	0	0	15
20-40%	0	0	6	6	0	7
0-20%	0	0	6	0	0	0

Table S4. Mean of quantified signal of detected cytokines of EB-derived rosettes and rosette neurospheres ± standard deviation (SD)

Examined Cytokines	EB-derived Rosettes N=25 each			Rosette Neurosphere N=5
	Category 1 (small)	Category 2 (Large single)	Category 3 (Medium)	
PDGF-AA	931.5 ± 898.2	722.6 ± 797.2	586.1 ± 727.4	25.8 ± 16.7
GDNF	254.2 ± 222.9	243.3 ± 163.3	146.3 ± 158.6	4.01 ± 8.9
IL-6	32.07 ± 43.1	31.1 ± 70.9	11.9 ± 23.4	0 ± 0
FGF-2	113.4 ± 165.1	58.5 ± 120.02	62.7 ± 108.3	23.1 ± 30.4
β-NGF	81.9 ± 107.6	21.8 ± 47.9	45.7 ± 81.2	8.7 ± 19.4
NT-3	57.6 ± 95.1	14.5 ± 42.7	21.03 ± 51.7	0 ± 0
CNTF	78.5 ± 96.9	26.1 ± 49.8	78.5 ± 96.9	0 ± 0
BMP-4	14.8 ± 30.6	2.4 ± 8.7	5.1 ± 14.2	0 ± 0
BDNF	44.3 ± 45.1	22.2 ± 29.1	28.3 ± 34.9	21.9 ± 37.6
IGF-1	0.7 ± 3.7	0 ± 0	0 ± 0	0 ± 0

Table S5. Signaling profiles of neural rosette subtypes in individual microchambers. Abbreviations refer to Cat 1: EB-derived small rosettes, Cat 2: EB-derived large rosettes, Cat 3: EB-derived medium rosettes, NS-R: neurosphere rosettes.

		PDGF-AA	GDNF	IL-6	FGF-2	NT-3	NGF	CNTF	IGF-1	BDNF	BMP-4
Cat 1	1/75	+	+	+	+	+	+	+	+	+	+
Cat 1.2.3	9/75	+	+	+	+	+	+	+		+	+
Cat 1	2/75	+	+	+	+	+	+	+		+	
Cat 3	2/75	+	+	+	+	+	+			+	+
Cat 3	1/25	+	+	+	+	+	+			+	
Cat 1,2	3/75	+	+	+	+		+	+		+	
Cat 1, 2, 3	5/75	+	+	+			+	+		+	
Cat 3	1/75	+	+	+	+			+		+	
Cat 1.2	3/75	+	+	+	+					+	
Cat 1	1/75	+	+				+	+		+	
Cat 3	1/75	+	+	+				+		+	
Cat 3	1/75	+			+		+	+		+	
Cat 2	1/75	+		+			+	+		+	
Cat 1.2	2/75	+	+	+		+					
Cat 2	2/75	+	+	+						+	

Cat 1,3	2/75	+		+				+		+	
Cat 1.2	2/75	+	+					+		+	
Cat 3	1/75	+		+			+			+	
Cat 3	1/75	+			+		+			+	
Cat 1,2,3	11/75	+	+	+							
Cat 3	1/75	+		+				+			
Cat 2,3	2/75	+	+								
Cat 1,3	2/75	+			+						
Cat 3	3/25	+						+			
Cat 3	1/25							+			
Cat 1,2	7/75	+									
Cat 2,3	3/75		+								

Table S6. Variation seen in quantified signal of detected cytokines of rosette neurospheres in individual chambers

Cytokines	Chamber 1	Chamber 2	Chamber 3	Chamber 4	Chamber 5
PDGF-AA	20.3	0	42.5	29.3	37.1
GDNF	20.1	0	0	0	0
Il-6	0	0	0	0	0
FGF2	14.6	27.3	73.5	0	0
NT-3	0	0	0	0	0
β-NGF	43.5	0	0	0	0
CNTF	0	0	0	0	0
IGF-1	0	0	0	0	0
BDNF	86.8	23.04	0	0	0
BMP-4	0	0	0	0	0