COMMUNICATION

Supplementary Information for:

Heparin-based, injectable microcarriers for controlled delivery of interleukin-13 to the brain

Lucas Schirmer[a], Chloé Hoornaert[b], Debbie Le Blon[b,c], Dimitri Eigel[a], Catia Neto[d], Mark Gumbleton[d], Petra B. Welzel[a], Anne E. Rosser[e,f], Carsten Werner[a,g], Peter Ponsaerts[b,c] and Ben Newland*[, a,d]

1. Materials and Methods

Microcarrier synthesis: 4-arm starPEG (Mw 10,000 Da) end-functionalized with amino groups was purchased from Jenkem Technology USA. All other chemicals, such as porcine intestinal heparin sodium salt (Mw 14,000 Da), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), sulfo-N-hydroxysulfosuccinimide (sulfo-NHS),, toluene and the stabilizing agent Synperonic PEP105, were purchased from Merck unless otherwise stated.
Microcarriers (MCs) were synthesized in a similar manner to that reported previously [61,62]. Briefly, the microcarriers were prepared by crosslinking the amino end-functionalized starPEG with EDC/sulfo-NHS-activated carboxylic acid groups of heparin at -80°C. 200 µL of hydrogel mixture was prepared with 11.11 mg of starPEG and 15.56 mg of heparin (1:1 molar ratio) and a ratio of EDC to sulfo-NHS of 2:1 (1.7 mg and 0.97 mg respectively) in MilliQ water. For fluorescently labeled microcarriers, up to 1% of the heparin in the hydrogel precursor solution was substituted by Atto 488 or Atto 633 (ATTO-TEC GmbH) labeled heparin to enable their visualization by fluorescent microscopy. An emulsion of the aqueous precursor solution in toluene was prepared by stirring and cooled down to -80°C by means of a cooling bath in order to create ice crystals within the aqueous droplets before curing of the hydrogel phase. Lyophilization of the frozen particles resulted in macroporous cryogel microcarriers, which could be stored as a dry powder. The microcarriers were washed three times with ethanol and two times with MilliQ water before being passed through a 180 µm filter (Merck) and re-lyophilized.

Microcarrier characterization: The microcarriers were visualized in their hydrated/swollen state by light microscopy (Olympus CK X 41) and fluorescence microscopy (Dragonfly spinning disc confocal laser microscope (SPCLM) (Andor Technology)). Therefore, fluorescently labeled microcarriers were suspended in phosphate-buffered saline (PBS) and placed into a glass-bottomed well plate for visualization. Microcarrier diameter and their pore size were analyzed using PoreSpy [63] and visualized with ParaView (Kitware). Microcarriers in their dried state were mounted on carbon adhesive scanning electron microscopy (SEM) stubs and sputtered with gold for 60 s at 40 mA (SCD 050 Sputter Coater, Balzers). SEM imaging was performed using an XL30 ESEM-FEG scanning electron microscope (Philips) under high vacuum with an accelerating voltage of 5 kV.
In silico modeling of the interaction between IL-13 and heparin
The interaction between IL-13 and heparin was modeled based on PIPER and the automated protein-protein docking server Cluspro [42,64] modified for the binding of heparin tetramers as described by Mottarella et al. [41]. The docking results were rendered using Pymol (Schrödinger, USA).
To visualize the shape memory properties of the microcarriers, they were injected through a glass capillary with an internal diameter of 140 µm. The injection was undertaken under a Leica SE4 stereomicroscope and imaged using a Phantom MIROEX2 camera taking 1000 images per second.

IL-13 loading and release analysis: For IL-13 loading, 1 mg (dry weight) of microcarriers was added into transwell filter inserts for a 24 well plate (0.4 µm pore size, PET insert (Merck) by creating a stock dispersion of the microcarriers in loading buffer (PBS with 0.1% ProClin 300 (Merck)) and dividing it accordingly into the inserts. The loading buffer was drained through the filter leaving the microcarriers in their semi-dry state. Loading solutions were prepared to contain either 100 ng or 500 ng of recombinant murine IL-13 (Peprotech) in 200 µl loading buffer, which was added into each insert containing the semi-dry microcarriers. This created the 100 ng/mg of microcarrier and the 500 ng/mg of microcarrier groups. The plates were sealed with parafilm and left at room

temperature for 24 hours. Filters containing IL-13 solutions alone (without microcarriers) were also left at room temperature for the same time as a control for the possibility of protein degradation during the loading time.
After the loading period, the plates were centrifuged at 200 g in order to collect the supernatant. The IL-13 loaded microcarriers were washed twice with 200 µL release medium (Dulbecco's Modified Eagle's medium (DMEM) with 1% bovine serum albumin (Merck) and 0.1% ProClin) to remove any non-specifically bound IL-13. Supernatant and washing solutions were collected, flash-frozen in liquid N_2 and stored at -80°C until analyzed. After washing, the microcarriers were covered with 1 ml release medium and incubated at 37°C. Samples were collected from the supernatant at intervals of 3, 6, 24, 96, 168, 336 and 504 hours and stored at -80°C until analysis of the IL-13 content via an enzyme-linked immunosorbent assay (ELISA) (mouse IL-13 ELISA Ready-SET-Go, eBioscience) according to the manufacturers' protocol. For microscopical analysis, IL-13 was labeled with Atto 647 (ATTO-TEC GmbH) according to manufacturers' instruction and incubated with the microcarriers for 24 h. Fluorescent microscopy images were taken every 15 min to assess the protein accumulation onto the microcarriers.

Effect of IL-13 release on bone marrow-derived macrophages in vitro: Macrophages were isolated by culturing the bone marrow collected from C57BL/ 6JOlaHsd mice (Envigo) in non-treated tissue culture vessels with IMDM medium supplemented with Panexin BMM (Pan Biotech) and 15 ng/ml M-CSF (Peprotech) for seven days. Tissue isolation was performed in accordance with institutional and state guidelines and approved by the Committee on Animal Welfare of Saxony. The differentiation of the cells was validated by staining for the murine macrophage surface marker F4/80 (BD Biosciences), and CD11b (BD Biosciences), resulting in a >97% pure culture of F4/80+/CD11b+ macrophages. Washed IL-13 loaded microcarriers (corresponding 1 mg dry microcarriers, 100 ng/mg of microcarrier group or the 500 ng/mg of microcarrier group) prepared as described in the former section, but without the use of ProClin 300, were added to inflammatory macrophages (M1) as illustrated in **Figure 4A**. Therefore, the cells were seeded in a 24 well plate (250,000 cells in 800 µL medium per well) and allowed to attach for 1 hour before being challenged with 10 ng/mL LPS (lipopolysaccharide, Sigma) together with 10 ng/mL IFNγ (Interferon γ, Peprotech) and adding the transwell filter inserts containing the loaded microcarriers in 200 µL medium. For the controls, filter inserts with empty microcarriers, filter inserts with 200 µL pure medium without microcarriers and filter inserts with 200 µL medium and 10 ng of IL-13 (free IL-13) that had been incubated overnight with the loading samples at room temperature without microcarriers were used.
After three days in culture, the total RNA was isolated from the macrophages using Direct-zol RNA Kits (Zymo) and converted using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). To quantify gene expression, the synthesized cDNA was measured using the Dynamo ColorFlash SYBR Green qPCR kit (Thermo Scientific) with the primer pairs according to Table 1.

Table 1. Primer pair used for gene expression analysis

	Forward	**Reverse**
YWHAZ (NM_011740.3)	AAGACAGCACGCTAATAATGC	TTGGAAGGCCGGTTAATTTTC
PPIA (NM_008907.1)	AGACGCCACTGTCGCTTTT	AGTTTTCTGCTGTCTTTGGAACTTT
Arg1 (NM_007482.3)	CTTCCAACTGCCAGACTGTG	TGTCCCTAATGACAGCTCCTTTC
Ym1 (NM_009892.2)	GCTGCTACTCACTTCCACAGG	GGGACTATTTTCTCCAGTGTAGCC

***Microcarrier mediated IL-13 delivery to the mouse striatum*:** Wildtype C57BL/6 mice were purchased from Charles River. During the entire study, mice were kept in the animal facility of the University of Antwerp under a normal day-night cycle (12/12) with free access to food and water. All experimental procedures were approved by the ethical committee for animal experimentation at the University of Antwerp (approval no 2015-46).
For mouse implantation experiments, dry microcarriers were resuspended at a concentration of 50 mg/mL in either sterile PBS (empty microcarriers) or PBS containing 1000 ng of IL-13 per mg of microcarrier and left at 4°C for four days prior to injection. All surgical experiments were performed under sterile conditions. 8-week old female C57BL/6 mice (n=7) were anesthetized by an intraperitoneal injection of a ketamine (80 mg/kg, Pfizer) and xylazine (16 mg/kg, Bayer Health Care) mixture in 0.9% NaCl (Baxter) and placed in a stereotactic frame (Stoelting). A midline scalp incision was made, and a hole was drilled in the skull using a dental drill burr (Stoelting) at 2.5 mm dextral from bregma. Next, an automatic microinjector pump (kdScientific) with a 10 µL-syringe (Hamilton) was positioned above the exposed dura. A 30-gauge needle (Hamilton), attached to the syringe, was placed through the intact dura to a depth of 2.3 mm. After 2 minutes of pressure equilibration, 3 µL of empty microcarriers (n=3) or IL-13 loaded microcarriers (n=4) suspension was injected. The needle was retracted after another 4 minutes of pressure equilibration to prevent the backflow of the injected microcarrier suspension. Next, the skin was sutured (Ethilon, Ethicon), and 100 µL 0.9% NaCl solution were administered subcutaneously in order to prevent dehydration while mice were placed under a heating lamp to recover.

Histological analysis: One week after implantation experiments, mice were deeply anesthetized with an intraperitoneal injection of 60 mg/kg pentobarbital (Nembutal, Ceva Sante Animale), transcardially perfused with 0.9% NaCl solution and fixed with 4% paraformaldehyde. Next, brains were surgically removed, post-fixed in 4% PFA, and freeze-protected with a sucrose gradient. Dehydrated brains were snap-frozen in liquid nitrogen, and 10 µm thick cryosections were made. Immunofluorescent staining was

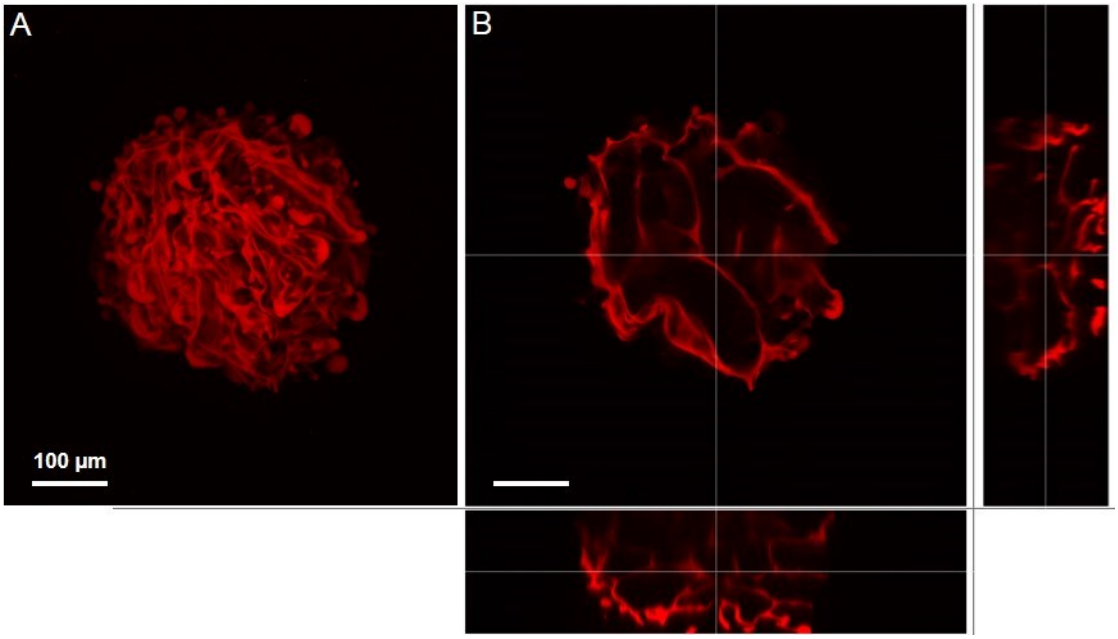
carried out according to previously optimized procedures [9] using the antibody combinations outlined in Table 2. Nuclear counterstaining was performed using TO-PRO-3 reagent (Thermo Fisher Scientific). Stained slides were mounted with Prolong Gold antifade reagent (Life Technologies), and images were acquired using an Olympus BX51 fluorescence microscope equipped with an Olympus DP71 digital camera. Olympus cellSens Dimension software was used for image acquisition and processing.

Table 2. Antibody (Ab) combinations (including supplier information) used for immunohistochemical analysis.

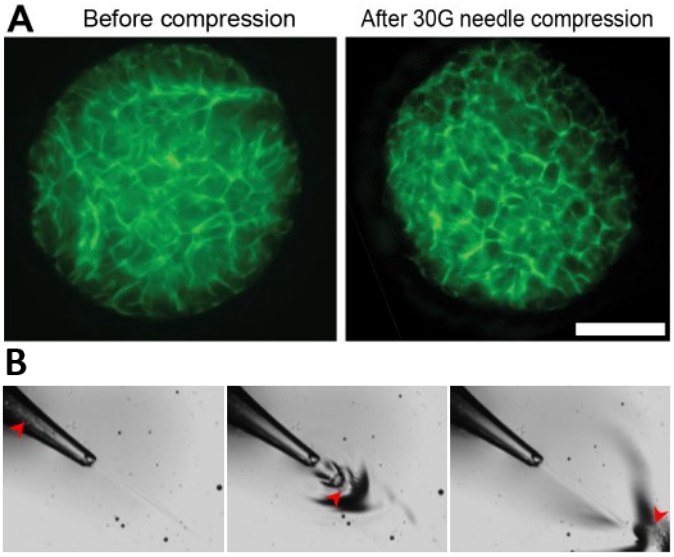
primary Ab	**dilution**	**company**	**secondary Ab**	**dilution**	**company**
mouse anti-GFAP	1:1000	Millipore (MAB360)	goat anti-mouse FITC	1:200	Jackson (115-095-164)
rabbit anti- IBA1	1:200	Wako (019-19741)	donkey anti-rabbit AF555	1:1000	Invitrogen (A31572)
rat anti-F4/80	1:250	AbD Serotec (MCA497GA)	goat anti-rat FITC	1:200	Jackson (112-096-143)
rat anti-MHCII	1:200	eBioscience (13-5321-85)	streptavidin-AF568	1:200	Invitrogen (S11226)
goat anti-ARG1	1:50	Santa Cruz (sc-18354)	donkey anti-goat AF555	1:200	Invitrogen (A21432)

Statistical analysis: One-way ANOVA with Tukey's post-hoc analysis was performed for the gene expression analysis using Prism (GraphPad Software). A student's t-test was performed using Excel software (Microsoft Corporation), to compare the differences between empty microcarriers and IL-13 loaded microcarriers, in terms of the percentage area of GFAP/Iba1/Arg1 immuno-staining. A *P*-value ≤ .05 was considered statistically significantly different.

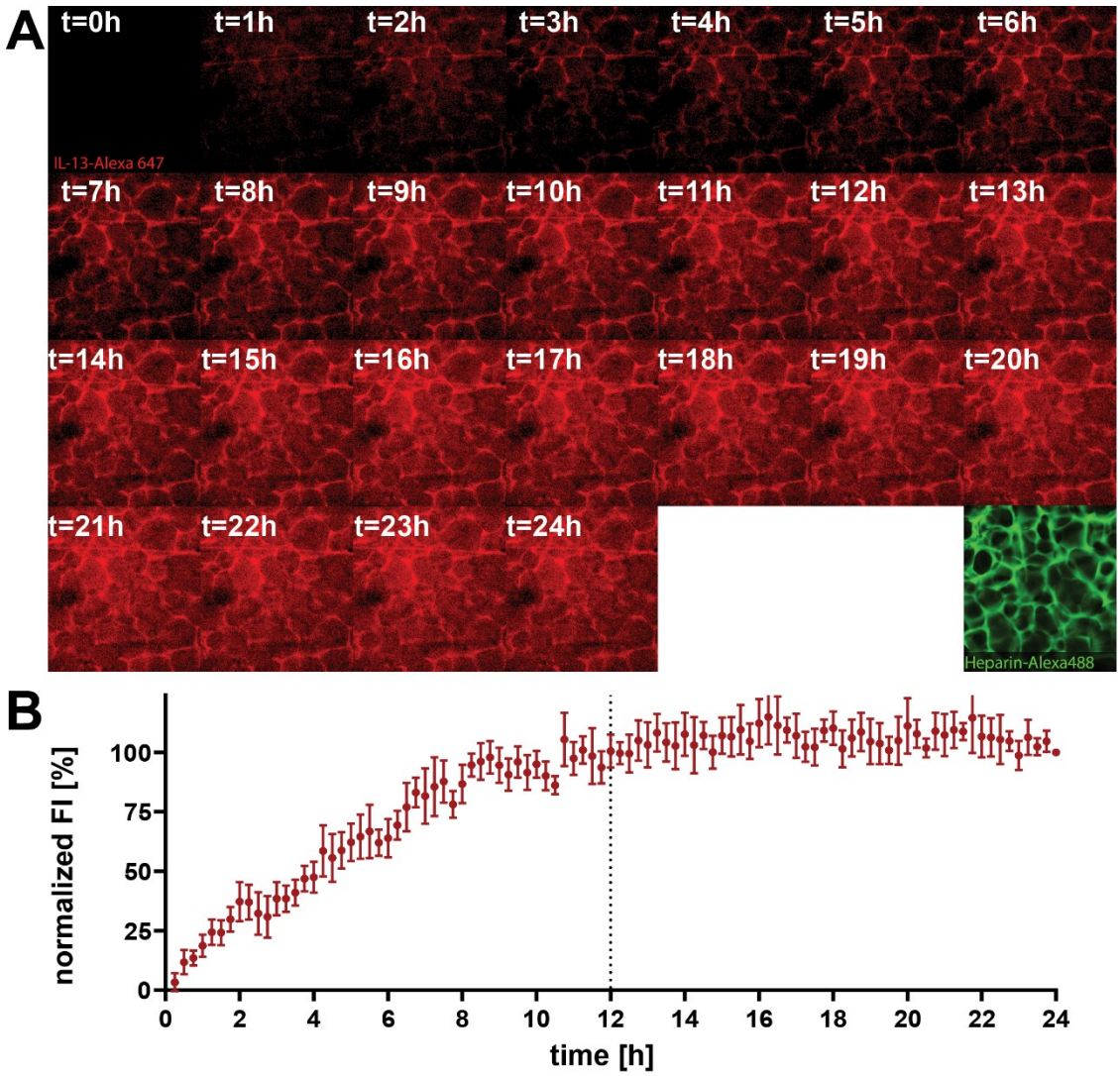
2. Supplementary Figures



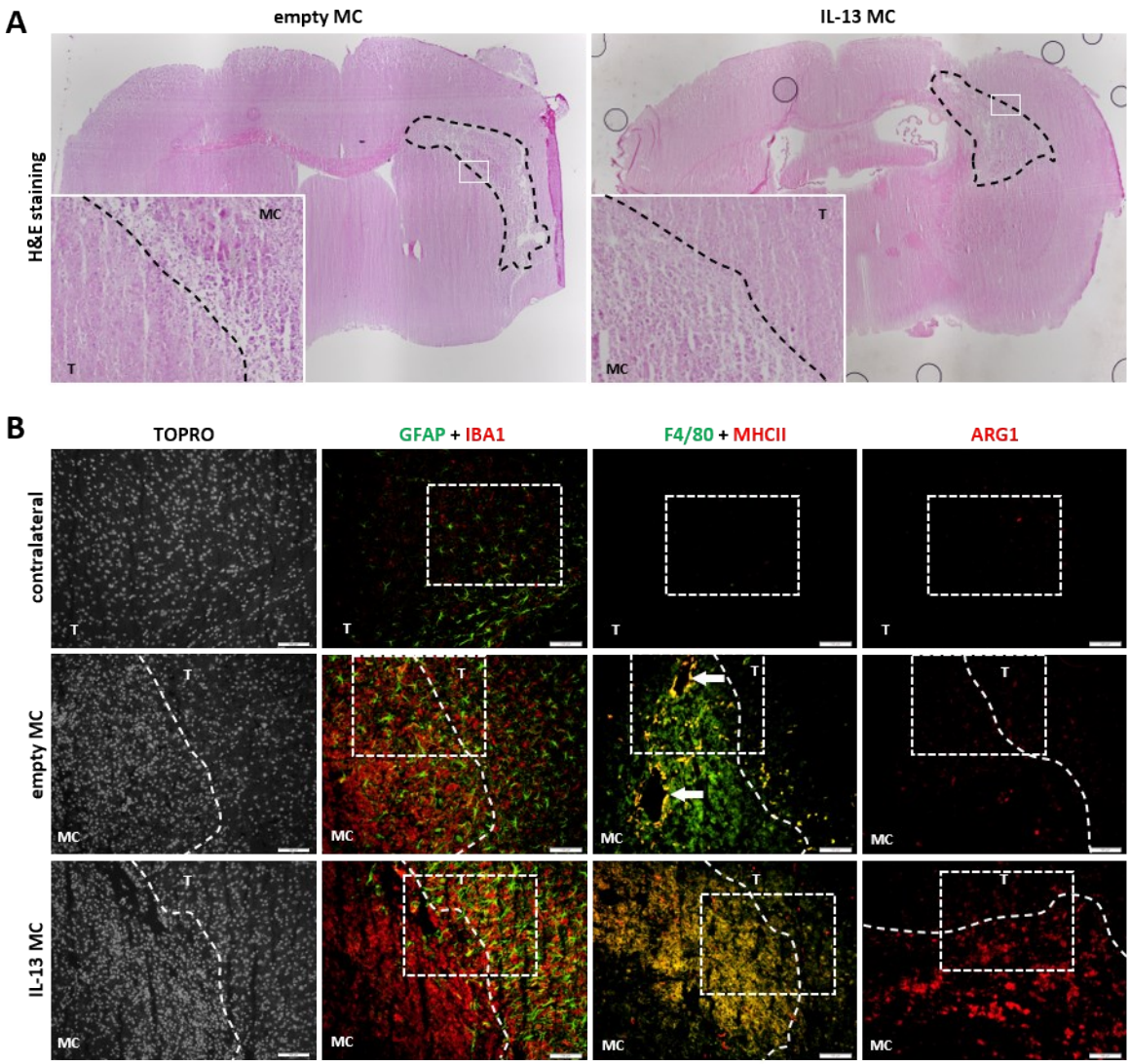
Supporting Information Figure S1. Confocal microscopy analysis of a microcarrier. A) shows an 80 µm z-plane projection image (z-stack). B) a single z-plane taken through the microcarrier, with corresponding images from the x and y axes.



Supporting Information Figure S2. Compressibility of cryogel microcarriers A) shows a z-plane projection image (z-stack) before and after compression through an 30G needle. Scale bar =200 µm. B) A time series of a 420 µm diameter microcarrier passing through a 140 µm glass capillary.



Supporting Information Figure S3. A) Confocal microscopy images of IL-13 uptake by a microcarrier over a period of 24 hours. B) Quantification of IL-13 fluorescence intensity (FI) within the microcarrier struts over time. (n=3, error bars represent ± standard deviation).



Supporting Information Figure S4. IL-13 released from microcarriers induces alternative activation of macrophages *in vivo*. Histological analyses were performed one week after in vivo delivery of empty microcarriers (Empty MC) (n=3) or IL-13-loaded microcarriers (IL-13 MC) (n=4). (A) Hematoxylin-eosin (H&E) staining reveals the MC injection area, outlined with a dashed line. MC = microcarrier, T = brain tissue. Low magnification images (B) Immunofluorescent staining for GFAP+ astrocytes, IBA1+ myeloid cells, F4/80+MHCII- activated microglia, F4/80+MHCII+ activated macrophages, and ARG1+ M2 polarized myeloid cells. TOPRO3 was used to counterstain nuclei. Scale bars =100 µm. White arrows indicate blood vessels, and dashed boxes show the region used in higher magnification for Figure 5 in the main text.

Supporting Information Movie S1. Possible binding sites of IL-13 to heparin shown in a 360 degree rotation. (separate file)