Electronic Supplementary Material (ESI) for ChemComm.
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Supplementary information

General experimental methods

Chemicals were obtained from Sigma Aldrich, Merck or Fisher Scientific, unless stated otherwise, and were of the highest purity available. Competent *E. coli* M834 (DE3) cells were from New England Biolabs. CdSe/ZnS core/shell nanoparticles (emission 600 nm) were purchased from PlasmaChem GMbH (Berlin) as a dry powder, capped with mixed ligands of trioctylphosphine oxide (TOPO), hexadecylamine and oleic acid. Other reagents were bought from the suppliers as listed in the sections below.

Size exclusion chromatography was performed on a GE Pharmacia ÄKTA FPLC system at 4 °C. Centrifugation was performed on either a Beckman Coulter Avanti J-30I or Heraeus Benchtop 3SR or Pico microcentrifuges. Spectrophotometric readings were measured using either a Thermo Scientific Nanodrop 2000 or WPA Biowave II spectrometer.

SDS-PAGE and agarose electrophoresis was performed using a Bio-Rad mini protean 3 apparatus and SCIE-PLAS Midi Horizontal Unit respectively. BIO RAD molecular imager^{R} Gel Doc^{TM} XR was used to visualise both the agarose and polyacrylamide gels using a combination of UV and white light. Protein concentrations were conducted using either a 10k or 30k MWCO Amicon® Ultra-15 Centrifugal Filter Device. Electrospray mass spectrometry was performed on a Bruker maXis Impact mass spectrometer and processed using the Bruker Compass Data Analysis maximum entropy deconvolution algorithm. Fluorescence spectroscopy was performed using a Cary Eclipse fluorometer. Microscopy equipment and procedures are detailed in the sections below.

Production of azido-CTB:

Plasmid pSAB2.2, described previously,[1] was subjected to Quikchange site-directed mutagenesis to create pSAB2.3_azido for expression of azido-CTB. Native methionine residues (excluding in the *E. coli* LTIIB leader sequence) were replaced with leucine residues (M37L, M68L, M101L), and a surface exposed lysine residue was mutated to methionine (K43M) for isosteric replacement with azidohomoalanine (Figure S1). The DNA sequence was confirmed (GATC Biotech) and the plasmid (pSAB2.3_azido) was transformed into *E. coli* M834 (DE3) Gold cells via heat-shock.

A single colony of the *E. coli* M834 strain harbouring pSAB2.3_azido was used to inoculate LB media (5 mL, 100 μg/mL ampicillin). The starter culture was incubated at 37 °C for 18 h before 2 ml was used to inoculate NMM media (0.5 L, 100 μg/mL ampicillin),[2, 3] which was then grown at 37 °C with agitation. Once the OD_{600} of the culture approached mid-log growth stage (1.25), the culture

was cooled to 30 °C and overexpression induced by addition of IPTG (1 mM final concentration) and azidohomoalanine (35 mg). Incubation was continued for 4 h at 30 °C before the cells were isolated by centrifugation (10000 × g, 10 min), the supernatant discarded and the cell pellet retained. The pellet was resuspended in phosphate-buffered saline (PBS; 10 mL) at 4 °C. The suspension was mechanically disrupted using a Constant Systems Cell Disruptor (20 kpsi, 10 mL injections, 4 °C) and the lysate was cleared by centrifugation (30000 × g, 45 min) before the supernatant was passed down a Nickel-affinity column (Qiagen, 10 ml) equilibrated in PBS. The column was washed sequentially with PBS (50 mL) and PBS supplemented with 20 mM imidazole (50 mL) before the protein was eluted with PBS containing 300 mM imidazole. Protein-containing fractions were identified by the Bradford assay and concentrated to a total volume of 1 mL by centrifugal concentration at 4 °C. The protein solution was applied to a size-exclusion column (HiLoad, Sephadex, 16/600 S75 column) and isocratically eluted in PBS before the purity of the isolated azido-CTB was analysed by SDS-PAGE and Electrospray mass spectrometry (Figure S2). The protein concentration was estimated by UV spectroscopy at 280 nm using a theoretical extinction molar coefficient of 11585 M^{-1} cm^{-1}.

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1     ACT CCT CAA AAT ATT ACT GAT TTG TGC GCA GAA TAC CAC AAC ACA   45
1     Thr Pro Gln Asn Ile Thr Asp Leu Cys Ala Glu Tyr His Asn Thr   15

46    CAA ATA TAT ACG CTA AAT GAT AAG ATC TTT TCG TAT ACA GAA TCG   90
16    Gln Ile Tyr Thr Leu Asn Asp Lys Ile Phe Ser Tyr Thr Glu Ser   30

91    CTA GCG GGA AAA AGA GAG CTG GCT ATC ATT ACT TTT ATG AAT GGT   135
31    Leu Ala Gly Lys Arg Glu Leu Ala Ile Ile Thr Phe Met Asn Gly   45

136   GCA ATT TTT CAA GTA GAG GTA CCA GGT AGT CAA CAT ATA GAT TCA   180
46    Ala Ile Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp Ser   60

181   CAA AAA AAA GCG ATT GAA AGG CTG AAG GAT ACC CTG AGG ATT GCA   225
61    Gln Lys Lys Ala Ile Glu Arg Leu Lys Asp Thr Leu Arg Ile Ala   75

226   TAT CTT ACT GAA GCT AAA GTC GAA AAG TTA TGT GTA TGG AAT AAT   270
76    Tyr Leu Thr Glu Ala Lys Val Glu Lys Leu Cys Val Trp Asn Asn   90

271   AAA ACG CCT CAT GCG ATT GCC GCA ATT AGT CTG GCA AAC TAA   312
91    Lys Thr Pro His Ala Ile Ala Ala Ile Ser Leu Ala Asn End   104
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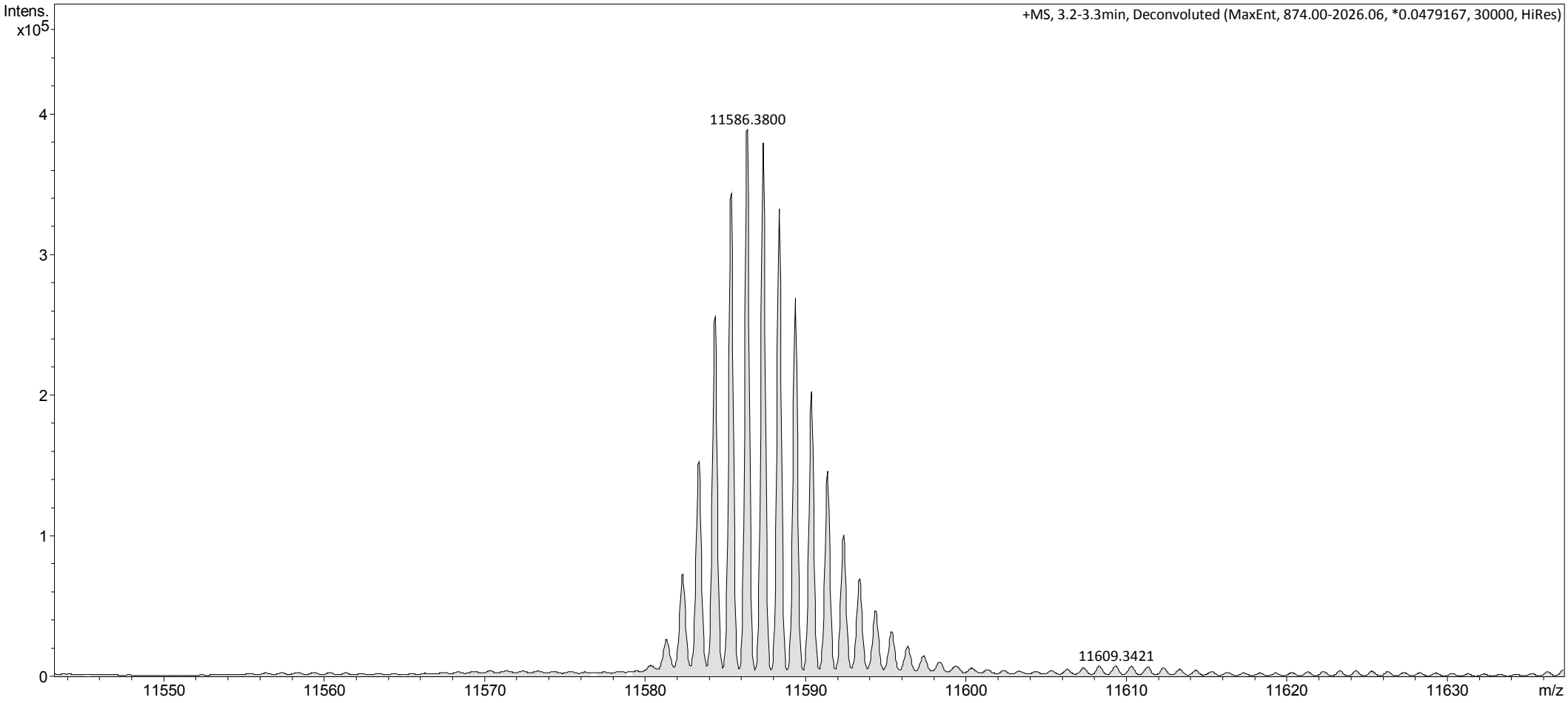
Figure S1. Sequence of the mutant CTB resulting from expression of pSAB2.3_azido. DNA and amino acid mutations introduced are highlighted in yellow. Met43 is replaced by its isostere azidohomoalanine when expressed in the presence of this non-canonical amino acid.

School of Chemistry Mass Spectrometry Service

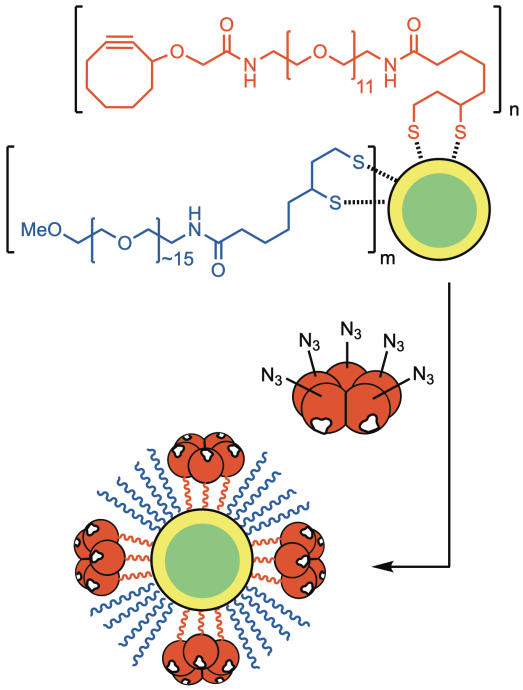
SampleID				**Submitter**	
Sample Description					
Analysis Name	D:\Data\bruceturnbull\chmdjwi\Azido CTB after S75_173945_RD7_01_25135.d			**Supervisor**	
Method	4b_AccMass_Protein_LC.m			**Acquisition Date**	05/08/2016 15:11:35
Instrument	maXis impact	**Source Type**	ESI	**Ion Polarity**	Positive
Scan Begin	250 m/z	**Scan End**	3000 m/z		



Bruker Compass DataAnalysis 4.1 Analysis Name Azido CTB after S75_173945_RD7_01_25135.d 15/08/2016 13:47:19 1 of 1

Figure S2. Deconvoluted mass spectrum of azido-CTB: calculated mass = 11586.0358

CdSe nanoparticle-conjugate synthesis: CdSe nanoparticle-(25% EG_{11}-cyclooctyne/75% PEG_{750}-OMe)



Ligand Reduction

Lipoic acid-PEG conjugate (LA-$PEG_{\sim 15}$-OMe; 2250 nmol)[4] and Lipoic acid-PEG_{11}-cyclooctyne (LA-PEG_{11}-cyclooctyne; 750 nmol)[5] were reduced with TCEP (3150 nmol) in aqueous MeOH (70 µL) at RT for 30 mins after which the solvent was removed. Chloroform (200 µL) and H_2O (200 µL) were added to the resulting solution and centrifuged at 21,100 x g for 1 min before the water layer was removed and centrifuged again.

CdSe/ZnS core-shell nanoparticle-Conjugation

CdSe/ZnS core shell nanoparticles (PlasmaChem GMbH, Berlin) capped with mixed ligands of trioctylphosphine oxide (TOPO), hexadecylamine and oleic acid (λ_{em} 600 nm, 1 nmol) in toluene (0.2 mL) were precipitated using ethanol (1.5 mL) and centrifuged at a speed of 10,000 x g for 5 mins. The clear supernatant was then removed and chloroform (50 µL) added to dissolve the pellet formed. This process was repeated twice more finishing on adding chloroform (100 µL) to the pellet. The reduced ligands DHLA-PEG_{11}-cyclooctyne (750 nmol) and DHLA-$PEG_{\sim 15}$-OMe (2250 nmol) dissolved in MeOH (150 µL) were added after deprotonation by NaOH in EtOH (0.10 M). The reaction was then stirred at RT in darkness for 30 mins. Hexane was added until the solution became cloudy. The mixture was then centrifuged at 15,000 x g for 5 mins obtaining the desired CdSe/ZnS nanoparticle pellet. The supernatant was removed and the pellet was dissolved in H_2O (100 µL). The solution was then transferred to a 30 kDa MWCO spin column and washed with H_2O (3 x 100 µL). A solution of the functionalised nanoparticles (10.66 µM, 33 µL) was mixed with azido-CTB (30 µM, 100 µL) and left at room temperature for 3 days.

CdSe/ZnS core-shell nanoparticle-conjugate synthesis: CdSe/ZnS core-shell nanoparticle-(10% EG_{11}-cyclooctyne/90% PEG_{750}-OMe)

Alternatively, the CdSe/ZnS core-shell nanoparticles were cap-exchanged with a 1:9 ratio of the LA-PEG_{11}-cyclooctyne and LA-PEG_{750}-OMe groups in an analogous manner starting with LA-$PEG_{\sim 15}$-OMe (2700 nmol) and LA-PEG_{11}-cyclooctyne (300 nmol).

Nanoparticle conjugates were characterised by agarose gel electrophoresis using a 0.75% agarose gel in Tris/Borate/EDTA (TBE) buffer 0.5x, pH 9.5 at 100 V as reported previously (Figure S3).[6] The gel shift in lanes 1-4 is consistent with successful protein conjugation to the nanoparticles.

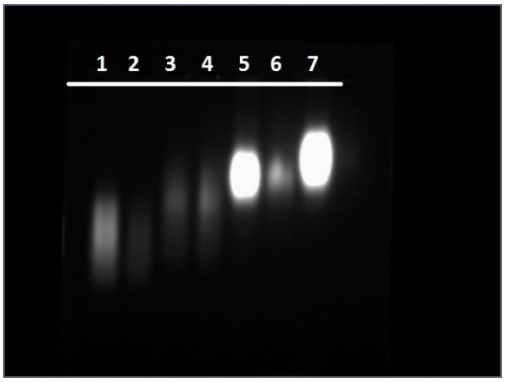


Figure S3: Agarose gel electrophoresis of QD-CTB *(25%)* lane 1 (5 µL), lane 2 (2 µL); QD-CTB *(10%)* lane 3 (5 µL), lane 4 (2 µL); QD-EG_{11}-Cyclo *(25%)* lane 5 (5 µL), lane 6 (2 µL); and QD-EG_{11}-Cyclo *(10%)* 7 (5 µL).

Absorbance and Fluorescence spectra of the CdSe/ZnS core-shell nanoparticles are shown in Figure S4. An excitation wavelength of 450 nm was used to record the emission spectrum.

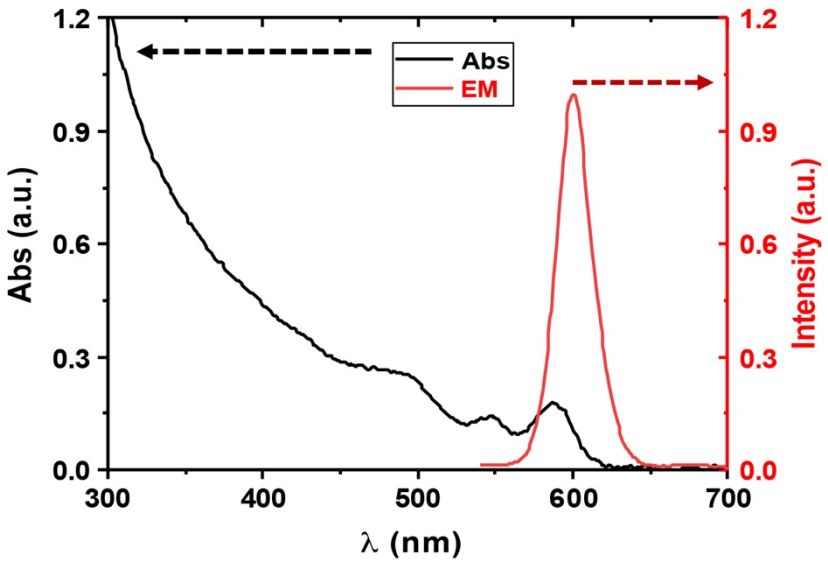


Figure S4: Absorption and emission spectra of the CdSe/ZnS core-shell nanoparticles in chloroform prior to cap-exchange. The cap-exchanged nanoparticles gave near identical spectra apart from the emission intensity being decreased by 5-10% in line with our previous observations.[7]

Tongue injections of Azido-CTB in mice:

All experiments were performed under Home Office Licence, in accordance with University of Leeds guidelines and the regulations of the UK Animals (Scientific Procedures) Act, 1986. Experiments were approved by the University of Leeds Animal Welfare and Ethical Review Board. Experiments were carried out on young C75Bl/6 adult mice (20–25 g) of both genders, bred in house.

Azido-CTB was tested for retrograde transport and detection with click chemistry *in vivo* via intramuscular tongue injections. Mice (n=4) were administered 50 µg of CTB-azide in 2.5 µL of 0.1 M PBS into the tongue with a glass micropipette attached to a 10 µL Hamilton syringe. Mice were perfused after two days by transcardial perfusion fixation with 4% PFA. The brainstem was removed, post-fixed in 4% PFA for 24 hours and then sectioned at 50 µm on a vibrating microtome.

Detection of Azido-CTB:

Brainstem slices were permeablised with 0.2% PBST for 30 minutes and then washed twice for 10 minutes in Tris buffered saline (TBS). A solution containing 100 mM Tris (pH 8.5), 1 mM $Cu(II)SO_4$, 10 µM biotinylated alkyne (EVU103, Kerafast) and 100 mM ascorbic acid was added to the wells, and left to incubate for 30 minutes at room temperature. The slices were then incubated with Streptavidin Alexa Fluor® 555 (Invitrogen). Controls of brainstem slices without azido-CTB underwent the click reaction to check for non-specific labelling.

To assess whether azido-CTB was labelled by the click reaction immunohistochemistry for CTB was also used. Sections were then blocked with 10% donkey serum for 30 minutes. Next they were incubated with rabbit anti-CTB (C3062, Sigma) diluted to 1:10000 in PBST (PBS with 0.1% Triton X-100) for 24 hours at 4 °C, followed by incubation in donkey anti-rabbit Alexa Flour ®488.

Sections were air-dried onto glass microscope slides and mounted under a coverslip with Vectashield mounting medium (Vector Labs). Slides were examined using a Nikon Eclipse E600 microscope equipped with epifluoresence and Q-Imaging Micropublishing 5.0 camera. Images were obtained using AcQuis image capture software and adjusted for brightness and contrast using CorelDraw x6 software.

Tongue injections of CTB-nanoparticles:

CTB-nanoparticles were tested for retrograde transport *in vivo* via tongue injections in mice. Mice (n=2) were administered 2 µL of CTB-nanoparticle (2.78 µM) into the tongue and perfused with 4% PFA with 1% glutaraldehyde after 3 days. The brainstem was removed, post-fixed for 24 hours and sectioned at 50 µm on a vibrating microtome. Mice were injected with just CdSe/ZnS core-shell nanoparticles as a control.

Detection of CTB-nanoparticles:

Brainstem slices underwent silver enhancement to visualise CdSe/ZnS-nanoparticles using HQ Silver (Nanoprobes). Manufacturer's guidelines were followed and slices imaged using Nikon Eclipse E600 microscope.

Some silver enhanced sections were further processed for electron microscopy (EM). Following silver enhancement slices were incubated in gold chloride to fix the silver. Following three 10 minutes washes in PB, slices were incubated in 0.5% osmium in 0.1 M PB for 30 minutes. Following another round of washes in PB and then in distilled water the slices were dehydrated in increasing concentrations of acetone for 7 minutes each (50%, 70%, 90%, 100%, 100%). Resin was then added in increasing ratios mixed with acetone (25%, 50%, and 80%) for 30 minutes each and then the slices placed in resin in a small metal planchette and left overnight for acetone to evaporate. A clean glass slide and the planchette are placed on a hotplate. Each section is removed from the planchette, placed onto the slide and mounted with a plastic coverslip. The slides are then incubated at 60 °C for 48 hours for the resin to polymerise. After removing the plastic coverslip the slide is heated and using a scalpel the resin around the slice scored until the slice is freed. The resin embedded slice can then be glued on top of a resin block and the excess surrounding resin trimmed away with a razor blade. Resin is then trimmed from the top of the block with a glass knife using the ultracutter. A diamond knife is used for cutting 70 nm sections of resin embedded tissue; the sections are collected copper slot grids coated with formvar. After drying the grids are incubated in uranyl acetate for 3 minutes. Ultra-thin sections are washed in distilled water and left to dry. The grids are then incubated in 1% lead citrate for 3 minutes and sections undergo a final wash in distilled water.

Finally, ultra-thin slices can be imaged using an FEI G2 Spirit with LaB6 gun equipped with Gatan 2k CCD camera, operating on 120kV. Images were put together using CorelDraw x6 software.

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