AAV9-Retro Mediates Efficient Transduction with Axon Terminal Absorption and Blood-brain Barrier Transportation

Kunzhang Lin  
Huazhong University of Science and Technology

Xin Zhong  
Wuhan Institute of Physics and Mathematics Chinese Academy of Sciences

Lei Li  
Wuhan Institute of Physics and Mathematics Chinese Academy of Sciences

Min Ying  
Wuhan Institute of Physics and Mathematics Chinese Academy of Sciences

Tian Yang  
Wuhan Institute of Physics and Mathematics Chinese Academy of Sciences

Zhijian Zhang  
Wuhan Institute of Physics and Mathematics Chinese Academy of Sciences

Xiaobin He (hexb@wipm.ac.cn)  
Wuhan Institute of Physics and Mathematics Chinese Academy of Sciences  https://orcid.org/0000-0001-8051-130X

Fuqiang Xu  
Huazhong University of Science and Technology

Methodology

Keywords: adeno-associated viruses, retrogradely, AAV9-Retro, across the blood-brain barrier, neural circuits

DOI: https://doi.org/10.21203/rs.3.rs-52147/v1

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Abstract

Recombinant adeno-associated viruses (rAAVs), especially which permit efficient gene transfer to neurons from axonal terminals or across the blood-brain barrier, are useful vehicles for the structural and functional studies of neural circuit, and for the treatment of many gene-deficient brain diseases that needs to compensate for the correct genes to every cell in the whole brain. However, AAVs with these two advantages have not been reported. Here, we describe a new capsid engineering method, which draws on advantage combination of different capsids, and aims to yield a capsid that can provide more alternative routes of administration, which are more suitable for wide-scale transduction of the CNS. A new AAV variant, AAV9-Retro, was developed by inserting the 10-mer peptide fragment from AAV2-Retro into the capsid of AAV9, and the biodistribution properties were evaluated in mice. By intracranial and intravenous injection in the mice, we found that AAV9-Retro can retrogradely infect projection neurons with efficiency comparable to AAV2-Retro, and retains the characteristic of AAV9 that can transport across the nervous system. Our strategy provides a new tool for the manipulation of neural circuits, and for the future preclinical and clinical treatment of some neurological and neurodegenerative disorders.

Introduction

Viral vectors, especially which permit efficient gene transfer to central nervous system (CNS) from axonal terminals or across the blood-brain barrier, are useful for analyzing structure and function of specific neuronal circuits around the injection site [1–11], and have become one of the most potential and promising therapy tools by delivery therapeutic genes to a distant target area [5, 12]. Compared with traditional retrograde tracers, viral vectors can express genes in specific neuron groups [9, 13], and have been widely used to monitor and manipulate neuronal activities by expressing optogenetic [14, 15], chemogenetic [16, 17] and calcium-sensitive functional probes [18–20]. Some natural and engineered neurotropic viruses exhibit retrograde infection capabilities, including pseudorabies virus (PRV) [21], herpes simplex virus (HSV) [22], rabies virus (RABV) [13, 23], lentivirus (LV) [24–27], canine adenovirus (CAV) [6, 28], and adeno-associated virus (AAV) [5, 29–31], etc. Among them, PRV is highly toxic [4, 32, 33]; HSV and RV can express genes rapidly and have high retrograde labeling efficiency, but they are also toxic to cells, limiting long-term gene manipulation [4, 13, 34, 35]. Rabies virus with double deletion of G/L has reduced the cyto-toxicity, but also gene expression [35]; Lentiviruses packaged by modified RVG have high retrograde transport efficiency [25], but may induce host immune responses [36, 37] or lead to tumorigenesis by random insertion into host genome [38]; CAV-2 has relatively low immunogenicity, large cloning capacity [39, 40], and enhanced retrograde labeling efficiency after compensation of the CAV-2 receptor in input neurons [6], but only moderate levels of gene expression [41] and difficulties in preparation [42].

Recombinant adeno-associated viruses (rAAVs) have become the powerful tool for neural circuit manipulation and gene therapy, as they are low-toxic with high-level transgene expression and minimal host immune responses [43–45]. Treatment of many gene-deficient brain diseases needs to compensate for the correct genes to every cell in the whole brain [44]. Some natural and engineered adeno-associated
viruses can transduce the majority of CNS neurons by axon terminal absorption or blood-brain barrier transportation, including AAV-TT [44], AAV2-Retro [5], AAV9-SLR [12], MNM004 [46], AAV-PHP.eB [47], and AAV-F [48], among others. However, AAVs with these two important features have not been reported. Compared with AAV2, AAV9 may have greater prospects for modification due to the advantages of lower neutralizing antibodies in humans [49], higher transduction efficiency in vivo and the ability to cross the blood-brain barrier [50, 51]. Therefore, AAV9 variants that provide efficient transduction by axonal spread and across the blood-brain barrier are needed. It has been reported that inserting the selected sequences from the AAV9 libraries into the capsid of AAV2 could not increase transduction efficiency compared with AAV9 variants, and vice versa in most cases [52]. Therefore, it is still unknown whether the peptide segments from AAV2-Retro (AAV2 libraries) can be integrated into the capsid of AAV9 to achieve high-efficiency retrograde labeling and to maintain the ability to cross the blood-brain barrier.

Here, we inserted the 10-mer peptide fragment from AAV2-Retro (AAV2 libraries) into the capsid of AAV9, and compared it with AAV2-Retro in neurotropism. By intracranial and intravenous administration in mice, we found that this new variant AAV9-Retro can retrogradely infect projection neurons with efficiency comparable to AAV2-Retro, and retain the ability to cross the blood-brain barrier. Our strategy provides a new retrograde tool for the manipulation of neural circuits, and for the future treatment of some neurological and neurodegenerative diseases.

**Materials And Methods**

**Animals**

Adult male (8-10 weeks old) C57BL/6 from Hunan SJA Laboratory Animal Company mice were used for all experiments. Mice were housed in an appropriate environment with 12/12 h light/dark cycle, water and food were supplied ad libitum. All surgical and experimental procedures were carried out in accordance with the guidelines formulated by the Animal Care and Use Committee of Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences.

**AAV9 Capsid Modification**

The 10-mer peptide sequence LADQDYTKTA from AAV2-Retro (Addgene plasmid # 81070) was inserted in between Q588 and A589 of the AAV9 capsid on the pAAV-RC9 (BrainVTA Technology Co., Ltd.) vector. The corresponding 30 bp DNA sequence was introduced into the AAV9 capsid by overlap-PCR, and cloned into the pAAV-RC9 vector via restriction enzyme BsiWI and NheI (New England Biolabs). The modified AAV9 capsid plasmids (AAV9-Retro) were verified by DNA sequencing.

**Recombinant AAV Vector Production**
The various AAV serotype vectors, including AAV2-Retro-CaMKIIa-EGFP, AAV9-Retro-CaMKIIa-EGFP and AAV2-Retro-CaMKIIa-mCherry, were produced by baculovirus-AAV expression vector system [53], and purified by iodixanol gradient ultracentrifugation [54, 55]. The purified rAAVs were titered by qPCR assay using the iQ SYBR Green Supermix kit (Bio-Rad), and diluted to $1.0 \times 10^{13}$ viral particles/mL.

**Administration of AAV Particles**

The stereotactic injection coordinates were selected according to Paxinos and Franklin's †The Mouse Brain in Stereotaxic Coordinates†, 4th Edition [56]. The stereotactic coordinates for VTA were: AP: -3.20 mm; ML: ± 0.45 mm; DV: -4.30 mm from the bregma; For CPu: AP: +0.38 mm; ML: ±2.00 mm; DV: -3.50 mm from the bregma. 8-10 weeks old C57BL/6 mice (20-25 g) were used for aav virus injection, and standard injection process was referred to previously reported method [11]. For single VTA site injection, mice were divided into two groups (N=3 in each group), 300 nl of rAAV2-Retro-CaMKIIa-EGFP, and rAAV9-Retro-CaMKIIa-EGFP viruses were infused into the VTA of each group, respectively; For mixed viral injection into VTA, rAAV2-Retro-CaMKIIa-mCherry and rAAV9-Retro-CaMKIIa-EGFP viruses were mixed at the particles ratio of 1:1 and injected into VTA at 300 nl; For single CPu site injection, mice were divided into two groups (N=3 in each group), 300 nl of rAAV2-Retro-CaMKIIa-EGFP and rAAV9-Retro-CaMKIIa-EGFP viruses were infused into the CPu region of each group, respectively. After 3 weeks of virus expression in vivo, the mice were sacrificed using conventional cardiac perfusion method. GFP-expressing rAAV9-Retro or rAAV2-Retro ($5 \times 10^{11}$ vg/mouse) were intravenously injected into adult mice, and the mice were sacrificed after 4 weeks of expression.

**Slice Preparation and Imaging**

The brains were soaked with 4% paraformaldehyde solution overnight, and dehydration was accomplished at 37 °C with 30% sucrose solution, the brain was sectioned with the thickness of 40 μm by frozen section machine, and the brain slices were taken at 200 μm intervals. Brain slices were washed 3 times with PBS, 5-10 minutes each time. After DAPI staining (diluted by PBS at 1:3000) for 10-15 minutes, the brain slices were washed with PBS 2 times and stucked neatly on the microscope slides, then sealed with 70% glycerol. Imaging was performed by using the Leica TCS SP8 confocal microscope (Leica, Germany) or the Olympus VS120 Slide Scanner microscope (Olympus, Japan).

**Statistical Analysis**

For cell counting, the images were divided into different brain regions with Adobe Photoshop CS5 according to Allen Mouse Brain Atlas (http://www.brain-map.org/). The EGFP-positive neurons were quantified by ImageJ software, and the cells from olfactory bulb were not counted. Data analysis was performed by unpaired t-tests using GraphPad Prism (version 5.01, San Diego, CA). Data were shown as
mean ± SEM. Statistical significance of differences was set at p<0.05. Graphs of virus injection were drawn using Sigma Plot (version 10.0, Systat Software Inc, San Jose, CA).

Results

Efficient retrograde connectivity tracing by rAAV9-Retrovariant

Previous report had shown that inserting the selected sequences from the AAV9 libraries into the capsid of AAV2 could not increase transduction efficiency compared with AAV9 variants [52], and the 7-mer PHP.B peptide (TLAVPFK) from the AAV9 libraries can improve transduction efficiency of AAV1 in vivo and in vitro when inserted in between S588 and T589 of AAV1 capsid [57]. Therefore, whether the peptide segments from AAV2-Retro (AAV2 libraries) can be integrated into the capsid of AAV9 to achieve high-efficiency retrograde labeling remains to be verified. To verify this, the 10-mer peptide sequence 

LADQDYTKTA from AAV2-Retro (AAV2 libraries) was inserted in between Q588 and A589 of the AAV9 capsid to produce AAV9-Retro (Fig 1A). rAAV-CaMKIIa-EGFP and rAAV-CaMKIIa-mCherry was packaged by using this AAV9-Retro (Fig 1B). In order to evaluate whether AAV9-Retro can achieve high-efficiency retrograde labeling input neurons, 300 nl of rAAV9-Retro-CaMKIIa-EGFP virus was infused into the VTA region, then local infection and cortical region projecting to VTA were imaged by confocal microscopy at 21 days post-injection (DPI). A large number of green fluorescent signals were found in VTA and cortex (Fig 1C), this result indicated that AAV9-Retro has high-efficiency retrograde access to input neurons.

rAAV9-Retro shows a similar retrograde gene transduction efficiency compared with rAAV2-Retro

rAAV2-Retro has higher retrograde transduction efficiency than AAV9 and plays an important role in the analysis of nerve circuits [5]. In order to compare the retrograde infection efficiency of rAAV9-Retro virus (based on AAV9 modification) with rAAV2-retro, the two viruses were injected into VTA sites (Fig 2A-2B), respectively. We found that the two viruses could infect the same upstream brain regions (Fig 2C-2J), including prefrontal cortex (PFC), somatomotor areas (MO), medial septal complex (MSC) and Midbrain reticular nucleus (MRN), etc, and there was no statistical difference in the total number of neurons projection to VTA (Fig 2K, 10770 ± 1394 for AAV2-Retro; 10278 ± 1957 for AAV9-Retro. P = 0.8477). These results indicate that rAAV9-Retro and rAAV2-Retro have the similar retrograde infection efficiency.

rAAV9-Retro and rAAV2-Retro can retrogradely label the same projection neurons through mixed injection

In order to verify whether rAAV9-Retro and rAAV2-Retro can retrogradely infect the same brain regions that project to the injection site and avoid the difference caused by injection site deviation or individual
variation of animals, rAAV2-Retro-CaMKIIa-mCherry and rAAV9-Retro-CaMKIIa-EGFP viruses were mixed at the particles ratio of 1:1 and injected into VTA at 300 nl (Fig 3A), local infection and projection regions were imaged by the Olympus VS120 Slide Scanner microscope at 21 days post-injection (DPI). Both green and red fluorescent signals appeared in VTA (Fig 3B-3D) and in the same brain areas that projection to VTA (Fig 3E-3P), including anterior cingulate area (ACA), somatomotor areas (MO), prefrontal cortex (PFC), agranular insular area (AI) and medial septal complex (MSC), etc, and most of them overlapped. These results showed that rAAV9-Retro and rAAV2-Retro can retrogradely label the same projection neurons, when mixed injection to the same brain area.

rAAV9-Retro and rAAV2-Retro exhibit similar retrograde infection tropism and efficiency at different brain regions

Viruses may have different infective characteristics and efficiency when injected into different brain areas. Therefore, we injected the two viruses into the brain area of Caudate putamen (CPu, Fig 4A and Fig 4E), which had been reported to mainly receive input from cerebral cortex (CTX), basolateral amygdalar nucleus (BLA) and thalamus (TH) via rAAV2-Retro [5]. We found that, like rAAV2-Retro (Fig 4B-4D), rAAV9-Retro could effectively infect these brain regions (Fig 4F-4H), and the efficiency was equivalent between them through quantitative analysis (Fig 4I, from CTX: 6495±479.8 for AAV2-Retro, 7119±507.6 for AAV9-Retro, P = 0.4219; from BLA: 737.3±19.19 for AAV2-Retro, 871±203.1 for AAV9-Retro, P = 0.5481; from TH: 1281±250.0 for AAV2-Retro, 945.0±256.6 for AAV9-Retro, P = 0.4010). Also, there was no statistical difference in the total number of neurons projection to CPu (Fig 4J, 8888 ± 672.8 for AAV2-Retro, 9366 ± 633.6 for AAV9-Retro, P = 0.6318). These results indicate that rAAV9-Retro and rAAV2-Retro have the similar retrograde infection tropism and efficiency at different brain regions.

rAAV9-Retro mediates efficient transduction across the central nervous systems after intravenous administration

Compared with AAV2, AAV9 has the advantage of transduction across the blood-brain barrier. In order to verify whether rAAV9-Retro can mediate efficient transduction across the central nervous systems, GFP-expressing rAAV9-Retro or rAAV2-Retro (5 × 10^{11} vg/mouse) were intravenously injected into adult mice, fluorescence signal in the brains of mice were imaged after 4 weeks of expression. We found that rAAV9-Retro allowed efficient transduction across the blood-brain barrier after intravenous administration, and eGFP signals were observed in several important brain regions (including cortex, striatum, pallidum, hippocampus, thalamus, hypothalamus and periaqueductal gray), which were not shown in rAAV2-Retro.

Discussion

Adeno-associated viruses play an important role in understanding the structure and function of the brain [58]. They are also the star vectors in the field of gene therapy, and can be used in the treatment of
various genetic defects, such as Parkinson [59], hemophilia [60, 61], and lysosomal disease [62], etc. Here, we have endowed the AAV9 capsid with efficient retrograde transduction capacity in mouse brain by inserting the 10-mer peptide sequence (LADQDYTKTA) from AAV2-Retro (AAV2 libraries) in between Q588 and A589. The retrograde infection tropism and efficiency of rAAV9-Retro is similar to that of rAAV2-retro in neural circuits. Importantly, rAAV9-Retro retains the ability to deliver genes across the blood–brain barrier.

Various neurotropic viruses, such as HSV1 [22], CAV2 [6], RABV [13], HiRet or NeuRet lentivirus [25], which have been widely used in the study of brain circuits, have the characteristics of retrograde access to specific or multiple types of neurons [63, 64]. However, high toxicity made them not conducive to long-term functional studies in model animals, which further limited their application in disease treatment [4]. Although nontoxic rabies virus has been developed, its expression ability is weaker and needs to expand the expression of foreign genes by carrying CRE and FLP recombinase combined with transgenic animals or AAV viruses [35]. Several AAV variants have been developed for more efficient transduction of nervous system. For example, rAAV2-Retro allows for efficient mapping, monitoring, and manipulation of projection neurons [5]; PHP.S, PHP.B and PHP.eB are engineered for efficient transduction across the peripheral and central nervous systems [47, 65, 66]; AAV-TT has greater diffusion capacity and better therapeutic effect than AAV9 [44], etc. AAV9 has lower neutralizing antibodies in vivo, and AAV9 variant can evade neutralization reaction more effectively than AAV2 or AAV2 variant in vitro [49, 52]. In addition, increasing the efficiency of AAV9 transduction and decreasing administration dosage can reduce the immune response caused by dose increase [67]. Moreover, compared with AAV2, AAV9 is less affected by the change of extracellular matrix in brain [12]. Therefore, it will be meaningful to compare the retrograde transduction efficiency between AAV9-retro and AAV2-retro in non-human primates.

Treatment of many gene-deficient brain diseases, such as mucopolysaccharidosis IIIC, needs to compensate for the correct genes to every cell in the whole brain [44]. AAV with effective retrograde infection or greater diffusion capacity can achieve a wider range of drug delivery. AAV9 has a unique advantage over other serotype viruses, that is, it can transport across the blood-brain barrier and target the whole brain by intravenous injection. The retrograde AAV based on AAV9 modification can also mediate efficient gene delivery to the brain by intravenous administration, and can be combined with local administration to achieve better therapeutic effect, which is also the next issue we need to study.

In summary, we have proved that AAV9-retro can mediate efficient transduction with axon terminal absorption and blood-brain barrier transportation, which provides an additional AAV vector tool for neuroscience and gene therapy.

**Abbreviations**

rAAV: recombinant adeno-associated virus; CNS: central nervous system; PRV: pseudorabies virus; HSV: herpes simplex virus; RABV: rabies virus; LV: lentivirus; CAV: canine adenovirus; VTA: Ventral tegmental area; PFC: Prefrontal cortex; MO: Somatomotor areas; MSC: Medial septal complex; MRN: Midbrain
reticular nucleus; **ACA:** Anterior cingulate area; **AI:** Agranular insular area; **CPu:** Caudate putamen (striatum); **CTX:** Cerebral cortex; **BLA:** Basolateral amygdalar nucleus; **TH:** Thalamus; **DPI:** days post-injection.

**Declarations**

**Ethics approval and consent to participate**

All procedures used were approved by the Animal Care and Use Committees at the Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences. All the experiments with viruses were performed in Biosafety Level 2 laboratory and animal facilities. Consent to participate: Not applicable.

**Consent for publication**

Consent for publication: Not applicable.

**Availability of data and materials**

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors here declare that there is no conflict of interest between them.

**Authors' contributions**

KL and FX contributed to the study idea and design; FX contributed to funding acquisition and resources; XH contributed to the supply of adeno-associated viruses; KL, XZ, LL, MY and TY performed the experiments and data acquisition; KL, XZ, LL, ZZ and FX accomplished data analysis; KL and FX drafted the manuscript, and contributed to review and editing. All authors read and approved the final manuscript.

**Funding**

This work was supported by the Key-Area Research and Development Program of Guangdong Province (2018B030331001), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB32030200) and the National Natural Science Foundation of China (31830035).
Acknowledgements

We thank Wenjing Fang from Wuhan Institute of Physics and Mathematics for guiding the intravenous injection, and Miss Pingping An and Yanqiu Li for keeping the mice.

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**Figures**
Efficient retrograde transduction by rAAV9-Retro (A) The 10-mer peptide sequence \textit{LADQDYTKTA} from AAV2-Retro (AAV2 libraries) was inserted in between Q588 and A589 of the AAV9 capsid to produce AAV9-Retro. (B) AAV-CaMKIIa-mCherry and AAV-CaMKIIa-EGFP vectors were used for packaging into viruses as a specific indicator of neurons. (C) Transduction validation of mouse brain neurons in vivo using AAV9-Retro. AAV-CaMKIIa-EGFP vector were packaged into AAV9-Retro virus, then 300 nl of rAAV9-Retro-CaMKIIa-EGFP virus was infused into the VTA region, then local infection and cortical region projecting to...
VTA was imaged by confocal microscopy at 21 days post-injection (DPI). A large number of green fluorescent signals were found in VTA and cortex. Scale bars = 1 mm. VTA: Ventral tegmental area.

Figure 2

Comparison of transduction efficiency between rAAV9-Retro and rAAV2-Retro injected into the VTA. (A-B) rAAV2-Retro-CaMKIIa-EGFP and rAAV9-Retro-CaMKIIa-EGFP viruses can efficiently infect neurons when injected into VTA sites, respectively. (C-J) The two viruses can infect the same upstream brain regions,

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including PFC, MO, MSC and MRN, etc. (K) Comparison of the total number of neurons projection to VTA using rAAV9-Retro and rAAV2-Retro. Data were shown as mean ± SEM. Statistical significance of differences was set at \( p < 0.05 \). There was no statistical difference in the total number of neurons projection to VTA (10770 ± 1394 for AAV2-Retro; 10278 ± 1957 for AAV9-Retro. \( P = 0.8477 \)). Scale bars = 200 \( \mu m \) for Fig A-B; Scale bars = 100 \( \mu m \) for Fig C-J. VTA: Ventral tegmental area; PFC: Prefrontal cortex; MO: Somatomotor areas; MSC: Medial septal complex; MRN: Midbrain reticular nucleus.

**Figure 3**

rAAV9-Retro and rAAV2-Retro can retrogradely label the same projection neurons through mixed injection (A) rAAV2-Retro-CaMKIIa-mCherry and rAAV9-Retro-CaMKIIa-EGFP viruses were mixed at the particles ratio of 1:1 and injected into VTA at 300 nl, local infection and projection regions were imaged by the Olympus VS120 Slide Scanner microscope at 21 days post-injection (DPI). (B-P) Both green and red fluorescent signals appeared in VTA (B-D) and in the same brain areas that projection to VTA (E-P), including ACA, MO, PFC, AI and MSC, etc, and most of them overlapped. Scale bars = 200 \( \mu m \). ACA:
Figure 4

Comparison of retrograde infection tropism and efficiency between rAAV9-Retro and rAAV2-Retro at different brain regions (A-H) The two viruses were injected into the brain area of CPu (A and E), which had been reported to mainly receive input from CTX, BLA and TH areas via rAAV2-Retro. Like rAAV2-Retro (B-D), rAAV9-Retro could effectively infect these brain regions (F-H). (I-J) Through quantitative analysis, we found that the efficiency was equivalent between them (I, from CTX: 6495 ± 479.8 for AAV2-Retro, 7119 ± 507.6 for AAV9-Retro, P = 0.4219; from BLA: 737.3 ± 19.19 for AAV2-Retro, 871 ± 203.1 for AAV9-Retro, P = 0.5481; from TH: 1281 ± 250.0 for AAV2-Retro, 945.0 ± 256.6 for AAV9-Retro, P = 0.4010). Also, there was no statistical difference in the total number of neurons projection to CPu (J, 8888 ± 672.8 for AAV2-Retro, 9366 ± 633.6 for AAV9-Retro, P = 0.6318). Data were shown as mean ± SEM. Statistical
significance of differences was set at \( p<0.05 \). Scale bars = 200 \( \mu m \). CPu: Caudate putamen (striatum); CTX: Cerebral cortex; BLA: Basolateral amygdalar nucleus; TH: Thalamus.

**Figure 5**

rAAV9-Retro can transduce neurons across multiple CNS regions after intravenous administration. GFP-expressing rAAV9-Retro or rAAV2-Retro (5 \( \times \) 10^{11} vg/mouse) were intravenously injected into adult mice. (A) Representative images of GFP signal in the brains of mice after 4 weeks of expression. (B) High magnification images of the brain sections shown in A. Scale bars = 500 \( \mu m \) for A and 200 \( \mu m \) for B.