nature biomedical engineering | PEER-REVIEW INFORMATION

Scalable and modular wireless-network infrastructure for large-scale behavioural neuroscience

Corresponding author: Jae-Woong Jeong

Editorial note

This document includes relevant written communications between the manuscript's corresponding author and the editor and reviewers of the manuscript during peer review. It includes decision letters relaying any editorial points and peer-review reports, and the authors' replies to these (under 'Rebuttal' headings). The editorial decisions are signed by the manuscript's handling editor, yet the editorial team and ultimately the journal's Chief Editor share responsibility for all decisions.

Any relevant documents attached to the decision letters are referred to as **Appendix #**, and can be found appended to this document. Any information deemed confidential has been redacted or removed. Earlier versions of the manuscript are not published, yet the originally submitted version may be available as a preprint. Because of editorial edits and changes during peer review, the published title of the paper and the title mentioned in below correspondence may differ.

Correspondence

Tue 25 Aug 2020
Decision on Article nBME-20-1707

Dear Prof Jeong,

Thank you again for submitting to *Nature Biomedical Engineering* your manuscript, "Scalable Wireless Network Ecosystems to Automate Behavioral and Circuit Neuroscience". The manuscript has been seen by 3 experts, whose reports you will find at the end of this message. You will see that although the reviewers have some good words for the work, they articulate concerns about the degree of support for the claims, and in this regard provide useful suggestions for improvement. We hope that with significant further work you can address the criticisms and convince the reviewers of the merits of the study. In particular, we would expect that a revised version of the manuscript provides:

*Demonstration of a high-throughput experiment with 20+ animals, or a synchronized study between the animal groups in different facilities, or both.

*Clarification of the ethical and security implications of running experiments simultaneously in different laboratories and countries.

*Demonstration of the performance metrics of the system such as reliability, error rates, bandwidth issues in bidirectional communication and power consumption.

When you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to the comments from all reviewers, the (revised, if needed) reporting summary, and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

* Clearly highlight any amendments to the text and figures to help the reviewers and editors find and

understand the changes (yet keep in mind that excessive marking can hinder readability).

* If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).

* If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).

* Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.

* The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).

* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We hope that you will be able to resubmit the manuscript within 25 weeks from the receipt of this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*. Because of the COVID-19 pandemic, should you be unable to carry out experimental work in the near future we advise that you reply to this message with a revision plan in the form of a preliminary point-by-point rebuttal to the comments from all reviewers that also includes a response to any points highlighted in this decision. We should then be able to provide you with additional feedback.

We hope that you will find the referee reports helpful when revising the work. Please do not hesitate to contact me should you have any questions.

Best wishes,

Michelle

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Dr Michelle Korda
Senior Editor, *Nature Biomedical Engineering*

Reviewer #1 (Report for the authors (Required)):

This manuscript describes a strategy to automate animal behavioral experiments in neuroscience studies. This ecosystem uses commercially available, low-cost wireless network and internet of things (IoTN) solutions, such as Bluetooth, ethernet and internet. The behavioral experiments are locally controlled with miniature computers, ie Raspberry Pi, communicate with locale network controller through Bluetooth and/or Wifi, and accessible via web browsers anywhere that has internet access. This system overall allows for the experiment set up remotely programmable. The authors claim this system enables remote, scalable, modular and chronic high-throughput neuroscience studies. The technical innovation of this work is in the system level design and integration of well-available devices and protocols (Raspberry pi, Bluetooth, etc). It's very capable in implementing several neuroscience study scenarios as demonstrated in the manuscript. However, it is arguable/questionable if this work
can live up with the aforementioned claims. The reviewer has the following comments and questions.

1.What unique value does this work deliver? Due to the limits of network latency and bandwidth, it is not realistic to remotely operate the experiments with real-time control (many real applications require millisecond latency) or to stream data (often require large bandwidth) to remote computers. The only useful function here is to 'reprogram' and 'monitor' the experiments. It is not convincing that this is truly valuable, nor was it convincing that this was challenging before this work. For example, commercial behavior setups have similar or better local scalability (16-64 controlled by one computer and scalable to hundreds), and a simple remote desktop seems to be able to do everything demonstrated here.

2.Is this system truly scalable? The manuscripts claim that system can scale up to 7 animals in a local piconet and to hundreds overall. All their demonstrations, however, were with no more than a few animals. In addition, none of these demonstrations had high demand in data rate or fast control, which in many applications often are both required. In reality, Bluetooth and wifi both have limited overall bandwidth, leading to severe interference when device number goes up. (we all have experience with bad connections of Bluetooth mouse/keyboard) The authors should the capability of this system to handle large data stream, fast control and multiple devices, and report the true limits rather than theoretical limits.

3.Were these experiments fully approved by the IACUC's in their institutions? These experiments involve researchers in a different institute/country to operate animal experiments and potentially broadcasting video streams of animal experiments if things go wrong. Were there special measures and trainings involved in these protocols?

4.Point 3 leads to the question regarding the overall security concerns of this strategy. Wifi, cellular network and Bluetooth are not sufficiently secure to guarantee the safety of the animal and the data. Besides, there are many uncertainties involved here. For example, are there trained technician required within the piconet while the experiments were remotely controlled? If not, what if human intervention is required suddenly? (For example, animal escape or fighting)?
What if a remote researcher initiated a potentially harmful stimulation/modulation, and his or her cell phone loses internet?
What if researchers in country A operate animal experiments in country B, but did not fully follow the protocol and caused animal welfare issues? Who will be responsible?

Reviewer #2 (Report for the authors (Required)):

The manuscript "Scalable Wireless Network Ecosystems to Automate Behavioral and Circuit Neuroscience" describes the design and deployment of a multi-specimen optogenetic stimulation system. The Authors present an affordable and scalable probe that can be implanted in a large number of animals, and that can be controlled using wireless network infrastructure dubbed "Internet of Things in Neuroscience." (IoTN) The system allows to control up to 7 devices using a single smartphone and more with the user base minicomputer. The minicomputers can be theoretically connected over the Internet which would allow to run simultaneous experiments in multiple separate laboratories. The high throughput neuroscience experiments are demonstrated in the mouse and rat models. The presented experimental methods are very clear and thorough, which is another strong side of this manuscript.

Described demonstration of high-throughput neuroscience experiments has significant importance and without a doubt will be stimulating to the field. Affordable, scalable and automated systems can enable new experimental designs that would otherwise be very troublesome for the human experimenter to oversee in person. However, even though the concept of IoTN is highly valuable, there is a certain overlap with the previous article by some of the Authors (Nature Biomedical Engineering volume 3, pages655–669(2019)) to substantiate publication of this manuscript in its current form. What can make this study stand out is the real demonstration of high-throughput experiment with 20+ animals and/or synchronized study between the animal groups in different laboratories/facilities/universities so that the Authors fully demonstrate the premise of IoTN.

Other comments:

1. While it is understandable that a lot of computer code can be reused, it should be very cautious about reusing figures. Supplementary Fig. 1 was conceptually very similar to the Supplementary Fig. 14 in Nature Biomedical Engineering, volume 3, pages 655–669 (2019). Supplementary Fig. 3 is Figure 3b in the previous article. The Authors should correct this and avoid similar issues in the future.

2. For an article to be accessible to the broad audience, I think that the authors should look carefully for computer science terms that might be very new, niche or confusing. I believe that the word "Piconet" does not really exist in common scientific parlance and should be introduced and explained. The Authors could

consult colleagues without expertise in computer science to identify other confusing terms.

3. Figures 1 and 2 feel very crowded, the Authors could try to reduce their complexity. Especially, in Fig. 2d(iii) I am unsure if all the peripheral elements of minicomputer are necessary on the scheme. Also, in Fig. 3c multiple abbreviations are used and “P” is never defined. Both of these figures could be made more condensed and clear.

4. How many devices can be connected per minicomputer? Is it only 7 or 7 per BLE dongle?

5. I find the sentence on p. 7: “We used this identification technique to enable Local Piconet mode to control devices simultaneously and selectively with 100% accuracy. “ little misleading because with 48-bit Bluetooth address space the chance of incorrect pairing is close to 0%. The Authors could clarify that this is a comparison to IR- or RF-controlled devices which do not offer digital fidelity of the Bluetooth technology.

Reviewer #3 (Report for the authors (Required)):

The authors report a novel wireless method to conduct neurophysiological experiments. The stated intent of the report is that this capability will allow one to remotely and independently control multiple behavioral/neurophysiological experiments simultaneously in rodents (or other animal models) involving optogenetic stimulation, drug delivery, electrical stimulation, sensing basic environmental conditions such as temperature, pressure, humidity or perhaps neurosensing (although no examples or data are suggested) etc. The authors propose a local network called a “piconet” using conventional Bluetooth low energy (BLE) technology which allows the control of multiple independent experiments within 100 m (range of Bluetooth systems). In addition, the authors propose a novel IoTN (Internet of Things in Neuroscience) method to integrate multiple piconets anywhere in the world through a cloud approach. Therefore, the IoTN can be used to control BLE through a cellular phone or access
the internet through Wi-fi or the cellular network and access internationally located piconets.

Summary of results - The local BLE will be useful in simultaneously controlling behavioral experiments involving a maximum of 7 animals using a local user. While the Wi-fi capability and webserver running on a local Raspberry PI minicomputer will allow the user to receive data from multiple locations around the world. So, in terms of hardware requirements, one needs only a mobile phone for local piconet setup (for a BLE) and an additional Raspberry PI minicomputer for worldwide access through Wi-fi. The devices (remote control modules or RCMs) will need a radio receiver or transceiver and a 48-bit unique address to communicate with the central RCC (remote control center) node within each piconet. The authors describe two modes of operation – the local piconet mode (or P mode) for local operation of the piconet and the global internet (or I mode) for accessing a remote cloud network that enables control and access around the world.

1.To demonstrate effectiveness, the authors use the local piconet and global internet to control the feeding behavior of AgRP expressing mice by stimulating the PVH (paraventricular nucleus of the hypothalamus) using micro-LEDs.

2.Simultaneous and selective control of multiple experiments through IoTN. Micro-LEDs were implanted in the motor cortex of 6 mice and were activated through local piconet to increase their locomotor activity.

3.The authors demonstrate selectivity through activation of 2 distinct opsins (inhibitory and excitatory) through optical stimulation of implanted micro-LEDs in the VTA of mice. Using amber and blue light they were able to either increase social interaction in a statistically significant way or decrease social interaction (not statistically significant).

4.To demonstrate the versatility of the IoTN, the authors demonstrate the ability to activate a microfluidic device to pharmacologically manipulate the food consumption of transgenic mice.

5.Finally, to demonstrate the ability to perform delayed scheduling of IoTN activated experiments (thereby eliminating the need for an experimenter to be present and arguably eliminating the ‘observer effects’), the authors schedule the activation of the arousal modulating properties of the locus coeruleus noradrenergic system using an fiber optic excitation of the pre-frontal cortex.

Innovation – BLE technology exists already and there is nothing in this report that appears to add to that capability. BLE in its current form can also operate in mesh configurations to significantly scale up the number of different experiments that can be controlled. The innovation in this report appears to be two-fold:
a.Addition of a capability to wirelessly access a cloud network through Wi-fi and webserver on a local minicomputer.
b.Characterization of the resultant IoTN system in 5 distinct neurobehavioral experiments, 4 of which involved micro-LEDs or optic fibers and one involved a microfluidic device.

Degree of advance - In my opinion, this is a methodology report. The degree of advance in the methodology proposed by the authors is incremental to the existing state-of-the-art in wireless communications.

Implications - The demonstrations in this study are too specific and too early. For a report on a new method, it has to be much more focused on the technology with a far greater rigor in design and testing than on the applications as this study does.

Major technical criticisms/weaknesses - The weakness of this study is its design and the lack of focus. It is essentially reporting the development of a new capability or tool, which the authors refer to as the IoTN. The authors argue for the need for such a tool in neurobehavioral experiments where there is a need to control multiple experiments remotely all over the world. Technology development reports such as these should clearly lay out the different metrics of performance and the quantitative requirements for each of these metrics that the proposed technology should satisfy. The system design should then be motivated by these requirements. However, in this report, the required metrics of performance and the corresponding quantitative requirements are not explicitly laid out. The whole design process has an ad-hoc feel to it. The experiments to test and demonstrate the capabilities of the technology are needlessly complicated. Simple bench-top experiments could have provided
much of the results that the authors are looking for, with far greater rigor.

The authors test and demonstrate this technology in 5 complicated neurobehavioral experiments with the unstated performance criteria such as – “effectiveness”, simultaneous control, selectivity, “flexibility”, ability to perform delayed scheduling of experiments. To develop a wireless technology to remotely sense and control neurobehavioral experiments (although no “sensing” was demonstrated in this study), there are several other performance metrics that are far more important and should be addressed carefully but are not addressed here such as - reliability, error rates, bandwidth issues in bidirectional communication, power consumption etc.

Minor technical criticisms
Reliability of IoTN – The authors use the piconet and global internet to control implanted micro-LEDs bilaterally in rodents. The authors report in Fig. 3b, a 0.75 ms latency using the local piconet and a 100 ms latency using the global internet. What was the RCM-RCC distance for this 0.75 ms latency and how does the latency, and error rate vary with RCC-RCM distance and the number of RCMs on the piconet? Also, there are several questions and concerns regarding the variabilities in the latencies with the global internet. Are these variabilities within each RCM-RCC pair over a period of time or are these variabilities across multiple RCMs?

Sun 27 Jun 2021
Decision on Article NBME-20-1707A

Dear Prof Jeong,

Thank you for your patience in waiting for the feedback on your revised manuscript, "Scalable Wireless Network Ecosystems to Automate Behavioral and Circuit Neuroscience". Having consulted with the original reviewers (whose comments you will find at the end of this message), I am pleased to write that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*, provided that the points specified in the attached instructions file are addressed.

When you are ready to submit the final version of your manuscript, please upload the files specified in the instructions file.

For primary research originally submitted after December 1, 2019, we encourage authors to take up transparent peer review. If you are eligible and opt in to transparent peer review, we will publish, as a single supplementary file, all the reviewer comments for all the versions of the manuscript, your rebuttal letters, and the editorial decision letters. **If you opt in to transparent peer review, in the attached file please tick the box 'I wish to participate in transparent peer review'; if you prefer not to, please tick 'I do NOT wish to participate in transparent peer review'.** In the interest of confidentiality, we allow redactions to the rebuttal letters and to the reviewer comments. If you are concerned about the release of confidential data, please indicate what specific information you would like to have removed; we cannot incorporate redactions for any other reasons.
More information on transparent peer review is available.

Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

Reviewer #1 (Report for the authors (Required)):

This revised manuscript "Scalable Wireless Network Ecosystems to Automate Behavioral and Circuit Neuroscience", by Prof Jeong and colleagues, has included additional data and statements to address major concerns regarding system scalability and stability, as well as animal welfare in this novel operation of IoT. These new data are sufficient and support the novelty of this work. The reviewer suggestions the acceptance of this work.

Reviewer #2 (Report for the authors (Required)):

The authors have fully addressed my previous comments (I have also checked the authors' response to the editor's comments). The publication is recommended.

Reviewer #3 (Report for the authors (Required)):

Summary of results - In addition to my earlier summary of results, the authors have now added an impressive amount of experimental characterization involving 20+ animals in two different experiments.

Degree of advance - In my opinion, this is a methodology report. One of the many strengths of this report that I like is the use of existing state-of-the-art in wireless communications, in designing and demonstrating a fairly broad range of neurobehavioral experiments. This makes the technology more accessible to other laboratories around the world.

Implications - The demonstrations in this study are specific and extensive. Their revisions have added significant rigor to the characterization. I think this technology/report can be widely disseminated if the codes and designs are made public or the authors' choose to commercialize their development. If that is done, it can significantly impact a range of neurobehavioral experimentation.

Rebuttal 1

Dear Referees:

We are very grateful for the constructive and positive comments and feedback from the Reviewers. We have prepared this revised manuscript closely following your suggestions. In this letter, we outline the changes that were made. Please find changes to the manuscript highlighted in yellow. Below *your comments are in italic and underlined*. Our responses are in regular Arial.

Sincerely,

Sangtae Ha, Jordan G. McCall & Jae-Woong Jeong

Editorial Comments:

- *Thank you again for submitting to Nature Biomedical Engineering your manuscript, "Scalable Wireless Network Ecosystems to Automate Behavioral and Circuit Neuroscience". The manuscript has been seen by 3 experts, whose reports you will find at the end of this message. You will see that although the reviewers have some good words for the work, they articulate concerns about the degree of support for the claims, and in this regard provide useful suggestions for improvement. We hope that with significant further work you can address the criticisms and convince the reviewers of the merits of the study. In particular, we would expect that a revised version of the manuscript provides:*
    - ***Comment 1**: Demonstration of a high-throughput experiment with 20+ animals, or a synchronized study between the animal groups in different facilities, or both.*
    - ***Comment 2**: Clarification of the ethical and security implications of running experiments simultaneously in different laboratories and countries.*
    - ***Comment 3**: Demonstration of the performance metrics of the system such as reliability, error rates, bandwidth issues in bidirectional communication and power consumption.*

Our response: We are very grateful for the kind and positive comments for our manuscript as well as the insightful critiques from the reviewers which helped us revise the manuscript in ways that can clarify demonstration of our novel wireless technology. We have addressed all such suggestions as detailed in our responses beneath respective sections, especially the concerns relating to the novelty and characterization of the work. Below, we briefly summarized our response to the condensed editorial comments:

- **Response to Comment 1: (Demonstration of a high-throughput experiment with 20+ animals, or a synchronized study between the animal groups in different facilities, or both)**

    To address the reviewer's comment regarding high throughput experimentation, we designed a behavioral experiment that integrated two of the previously described behavioral assays with many more additional animals. In this case, we simultaneously conducted feeding and locomotor assays, coordinating stimulation protocols using internet-controlled scheduled stimulations. Here, we scheduled the delivery of two distinct commands to 8 Thy1-ChR2 animals to stimulate locomotor behavior in one experimental setting while simultaneously delivering commands to 13 AgRP-ChR2 animals in a separate setting to stimulate feeding behavior. As demonstrated in Figure 5, we successfully coordinated two completely different experiments via internet by simultaneously scheduling commands to 20-21 animals depending on the experimental conditions. We performed both of these experiments twice, once with automated feeding measurements (Fig. 5) and once with manually weighed food (Supplementary Fig. 9). These studies provide clear evidence demonstrating the possibility of conducting high-throughput and multiple simultaneous experiments with diverse behavioral assays in 20+ animals.

- **Response to Comment 2: (Clarification of the ethical and security implications of running experiments simultaneously in different laboratories and countries)**

  These animal experiments were fully approved by each institution's IACUC. Each behavioral experiment was conducted in accordance of IACUC approved protocols and managed by experimenters with years of behavioral assay experience. While our IoTN system enables fully-remote and global access, it is important to note that none of the experiments in this manuscript were performed without the necessary human oversight to ensure animal safety. Our laboratory is purposely set up with local remote (outside of the behavioral procedure rooms) video access so that experimenters can monitor animal behavior without direct interaction with the animals. These video feeds aid in designing careful experimental design, but also provide onsite investigators with vastly more information about animal welfare than is typical in most closed-box behavioral experiments. While all experimental modifications were monitored closely by personnel onsite, the environmental sensing can be safely achieved remotely and this study was conducted from ~350 km outside of the laboratory. However, because these studies take place in the homecage, animal welfare was ensured through daily animal husbandry health and safety checks as with any other homecage in our colony. Furthermore, Our IoTN system requires user credentials for access, and thus only authorized users can access and conduct the experiment. The HTTP connection from a remote researcher to the IoTN system is encrypted using SSL (Secure Socket Layer), and so eavesdropping on the communication channel is not possible. In addition, the IoTN system can easily support IPSec (Internet Protocol Security), creating site-level security by connecting a remote network to a local network. We have added cautionary text to the discussion to address limiting access of the IoTN to highly trained users and those with sufficient animal research experience. This section of the discussion also suggests caution when planning remote experiments and address security concerns as well as promoting rigorous personnel and experiment vetting prior to implementing an IoTN approach.

- **Response to Comment 3: (<u>Demonstration of the performance metrics of the system such as reliability, error rates, bandwidth issues in bidirectional communication and power consumption)</u>**

  Following the reviewer's suggestion, we further characterized the IoTN system to demonstrate the performance metrics of the system as follows:

  1. **Error rate vs Distance** (**Supplementary Fig. 6a**): Once a single RCM (Remote Control Module) is paired with RCC (Remote Control Center), the IoTN system showed a 100% of success rate regardless of the distance between RCC and RCM.

2. **Latency vs Distance** (**Supplementary Fig. 6b**): The distance between RCC and RCM barely affects the latency of triggering the devices in both Piconet and Internet. Note that the latency in the global internet control increases due to a signal propagation delay between the remote user and RCC, but this delay can be eliminated by implementing the 'scheduled control', which uses a local network at pre-defined times.
3. **Error rate vs the Number of devices** (**Supplementary Fig. 6c**): Error rate of internet IoTN control network shows almost no failure (i.e., nearly 100% of success rate) regardless of the number of connected devices. On the other hand, while piconet IoTN mode shows perfect reliability (i.e., exactly 100% of success rate) when equal to or less than 15 RCMs are connected, the excess numbers of RCMs fail in operation when more than 15 RCMs are connected.
4. **Latency vs the Number of devices** (**Supplementary Fig. 6d**): The latency of the first operated device is almost the same regardless of the number of connected devices in both piconet and internet IoTN modes. In piconet IoTN mode, the "smartphone app" sequentially transmits the signals to the devices, which results in gradually increased latencies among the connected devices. On the other hand, in local network and internet IoTN modes, latency signals are sequentially transmitted from multiple "BLE dongles" to the connected RCMs, resulting in the variabilities of latencies among RCMs connected to the same BLE dongle. Due to these sequentially transmitted signals, in general, the latency to operate all the connected devices increases as the number increases. At a large scale, the effect of latencies can be partly mitigated by utilizing Bluetooth mesh networks where data can be transmitted through multiple pathways simultaneously in a large network.
5. **Latency vs Local/Global Internet control modes** (**Figs. 3b** and **c** and **Supplementary Figs. 6b** and **d**): The latency in the global internet control mode is larger than the local internet control mode due to a signal propagation delay between the remote user and RCC. However, this delay can be completely removed by implementing the 'scheduled control' mode, which solely uses a local network at pre-defined times and therefore capitalizes on its reduced latencies.
6. **Power consumption**: Since most of the IoTN hardware is powered through wall-sockets except the wireless RCMs, we characterized the power for the wireless RCMs only. We found out that the power consumed by the wireless RCM at average was 39.6 mW when the μ-ILEDs are operated at 20 Hz frequency with a 10 ms pulse width. Hence the battery used in wireless IoTN RCMs was selected to be 12 mAh for use in mice brains to allow over 60 minutes of uninterrupted wireless operation. The battery requirement of wireless RCM can be varied based on the length of the required experiment and the size of the animal.
7. **Bandwidth in bidirectional communication**: We demonstrated bidirectional sensing capability in our paper where we sensed temperature, pressure and humidity levels in the experimental home cages using the IoTN hardware and software. We believe that with further advancements in IoT communication technologies, a high bandwidth full-duplex stimulation (such as optogenetic and pharmacological) and neural activity monitoring (such as electrophysiological recordings and wireless calcium imaging) should be possible in the near future.

In addition, we added **Table 2** that summarizes the performance metrics as follows:

Table 2. Performance metrics of IoTN system

IoTN Performance Metrics	Local Piconet mode	Local Internet mode	Global Internet mode
Latency	~20 ms (max ~50 ms with 15 devices)	~55 ms (max ~90 ms with 22 devices)	(~55 + α) ms; where α = propagation delay (max ~ (95 + α) ms with 22 devices)
Error rate (= Misses/Trials x 100)	0% (with ≤15 devices)	<0.2% (regardless of number of devices)	<0.5% (regardless of number of devices)
Control distance between user and RCM	<100 m	No Limit	No Limit
The Number of controllable devices	15	22+ (100+ with Bluetooth Mesh)	22+ (100+ with Bluetooth Mesh)
Power consumption of wireless module	Tens of mW for μ-ILED operation	Tens of mW for μ-ILED operation	Tens of mW for μ-ILED operation
Bandwidth	~1 Mb/s (Bluetooth Low Energy, extendable up to 2 Mb/s for Bluetooth 5)	~1 Mb/s (Bluetooth Low Energy, extendable up to 2 Mb/s for Bluetooth 5)	~1 Mb/s (Bluetooth Low Energy, extendable up to 2 Mb/s for Bluetooth 5)

Reviewers Comments:

REVIEWER 1

- ***Summary Recommendation:*** *This manuscript describes a strategy to automate animal behavioral experiments in neuroscience studies. This ecosystem uses commercially available, low-cost wireless network and internet of things (IoTN) solutions, such as Bluetooth, ethernet and internet. The behavioral experiments are locally controlled with miniature computers, ie Raspberry Pi, communicate with locale network controller through Bluetooth and/or Wifi, and accessible via web browsers anywhere that has internet access. This system overall allows for the experiment set up remotely programmable. The authors claim this system enables remote, scalable, modular and chronic high-throughput neuroscience studies. The technical innovation of this work is in the system level design and integration of well-available devices and protocols (Raspberry pi, Bluetooth, etc). It's very capable in implementing several neuroscience study scenarios as demonstrated in the manuscript. However, it is arguable/questionable if this work can live up with the aforementioned claims. The reviewer has the following comments and questions.*

Our response: We appreciate the reviewer's comments. We have made modifications to reflect the reviewer's comments as described below.

- ***Comment #1:*** *What unique value does this work deliver? Due to the limits of network latency and bandwidth, it is not realistic to remotely operate the experiments with real-time control (many real applications require millisecond latency) or to stream data (often require large bandwidth) to remote computers. The only useful function here is to 'reprogram' and 'monitor' the experiments. It is not convincing that this is truly valuable, nor was it convincing that this was challenging before this work. For example, commercial behavior setups have similar or better local scalability (16-64 controlled by one computer and scalable to hundreds), and a simple remote desktop seems to be able to do everything demonstrated here.*

Our response: We thank the reviewer for the comment. At first glance, the IoTN technology might seem like incremental and a straightforward integration of off-the-shelf components and protocols with applications comparable to some commercial behavior setups used in neuroscience laboratories. However, instead of "a remotely controlled software with local off-the-shelf hardware", the IoTN technology is in real-sense a wholistic, customizable wireless ecosystem with the following novel values:

a. **Minimalistic hardware setups with unique wireless neural devices:** This not only includes off-the-shelf minicomputers, Bluetooth dongles and conventional equipment but also our untethered, modular, rechargeable and wirelessly programmable IoTN control modules as well as soft, bio-compliant and multimodal neural probes. Together these accessible technologies offer opportunities for untethered, chronic *in vivo* studies such as wireless optogenetics, drug delivery/optopharmacology, and environmental monitoring. Our wireless IoTN control modules can be easily swapped with different types and sizes of neural probes as well as different animals and/or other commercial equipment, thus enhancing overall versatility to neuroscientists with limited resources.
b. **Customizable and optimized software:** The software infrastructure here not only includes a globally accessible web user interface and a custom-designed smartphone app, but also our custom-defined communication architecture and driver infrastructure for customizable data packets, real-time data multiplexing and adaptable data streaming enabling unmatched scalability to a large number of devices. The protocols, data structures and communication infrastructure we separately engineered and

continuously optimized over a period of three years for their optimal integration, maximum reliability (nearly 100% success rate), minimal latency (~20 ms for the piconet and (~55 + α) ms for the internet control; α = global signal propagation delay) and seamless functionality together as a multi-wireless system. Note that the propagation delay (α) can be completely eliminated and ignored if using the scheduled control, where RCC uses only the local network.

c. **Novel applications for *in vivo* studies:** By optimally integrating the versatility and modular construction of both unique IoTN hardware and software within a single ecosystem, the IoTN technology is able to complete unique applications which have not been demonstrated before by a single standalone technology. This includes, but is not limited to low cost (~$45) and minimalistic hardware, global remote access, unmatched selectivity and scheduling (both at device level and at multimodal output level), customizable automation and high-throughput scalability. Upon its mass deployment, there can be new avenues for global collaboration within the scientific community, especially in developing countries that lack significant infrastructure and funding to carry out complex or large-scale studies.

Here we summarize the 14 key features offered by the standalone IoTN ecosystem to help significantly advance the boundaries of neuroscience research:

1. **Remote 24-hour Access**: Researchers are able to trigger, customize, stop, validate and store the outcomes of an experiment at anytime from anywhere in the world with an internet connection.
2. **Ease of use**: Our minimalistic hardware is easy to setup up even by non-engineers after a single demonstration. On the other hand, the frontend of our software user interfaces for both Global Internet (website) and Local Piconet (smartphone) were carefully optimized for simplicity and hassle-free operation after extensive iterative processes with established neuroscientists for many years.
3. **Multiple User Modes**: The IoTN technology offer two different control modes to the researcher depending on availability and experimental needs. The Local Piconet IoTN mode enables rapid creation of local on-the-fly networks with minimal effort and complexity. On the other hand, the Global Internet IoTN mode can be used as a global collaborative tool across multiple laboratories where researchers can exploit its remotely controlled automation and scheduling capabilities.
4. **Scheduling and Automation**: Researchers can schedule one or more experiments in one or more laboratories where each experiment can be configured with different unique animals as well as specific stimulation parameters within each device. Furthermore, by creating periodic scheduling commands, researchers can essentially automate multiple experiments at a time to create auto-generating data pipelines. All this can be achieved using a smartphone or browser and can be customized in real-time and verified later to validate its reliability.
5. **Selective Simultaneous Control:** Researchers can accurately and precisely select a specific group of animals or specific group of devices/device functions, alleviating the line of sight (LOS) and directionality handicaps prominent in contemporary wireless neural devices. Moreover, the selected devices and outputs can be simultaneously triggered or scheduled to a specific time which allows IoTN technology to perform selective simultaneous control within a large animal group or across multiple experiments and experimental groups.
6. **Bidirectional Communication:** The IoTN technology can transfer data not only from remote user to an end device but also from a remotely located end device with IoTN access to a user. Though current demonstrations include communication of sensor data such as pressure, temperature and humidity, further advancements in bandwidths and optimized latencies in wireless technologies can help integrate real-time neural recording and calcium imaging to the IoTN ecosystem.

7. **Potential for Mass Deployment for Scalable, Throughput Experiments:** One of the significant advantages of IoTN technology is its potential for mass deployment, especially when considering its minimalistic hardware, setup costs, ease of use and customizable versatility. Scientists across the world can quickly deploy this technology within their existing infrastructure with minimal budget concerns to achieve scalable experimental outcomes and data throughputs.
8. **Minimalistic Hardware:** A minicomputer and existing conventional equipment or wireless IoTN neural probes are all that is needed to establish a successful IoTN system. Comparing this with contemporary wireless control systems such as RF system where a single piece of equipment for single animal control can easily cost over $10,000 clearly highlights the significant economic advantage and versatility of IoTN ecosystem for selective simultaneous control of *in vivo* studies at a large-scale.
9. **Modular Hardware**: The tiny IoTN control modules offer quick swapping ability both with a rechargeable battery as well as an implantable neural probe. Based on experimental needs of the researcher, such as size of the animal, length of experiments, target neuronal stimulation and/or parameters, scientists can easily swap battery capacities and plug them into neural probes (in different animal brain sizes, different probe modalities) for efficient and economical utilization of minimalistic IoTN hardware resource. Furthermore, the IoTN control module can also be used to control various existing tethered equipment in neuroscience laboratories offering them instant IoTN access and wireless capability.
10. **Works with existing conventional tethered hardware**: Simply plugging our custom IoTN control modules (BLE modules) to conventional tethered equipment in already established neuroscience laboratories can help them achieve wireless functionality and IoTN access with minimal effort and resources. While the focus of this manuscript has been on neuroscience-related technologies, the IoTN ecosystem described here could easily be ported to any other field. In particular it is ready for deployment on any system using transistor-transistor logic to trigger device functions, or, of course, any device that can receive Bluetooth commands.
11. **Inexpensive and accessible technologies**: By linking design to readily available commercial hardware (Raspberry PIs and BLE dongles), widespread adoption of these technologies will be enabled for hundreds of dollars compared to other technologies that easily run in the tens of thousands of dollars.
12. **Optimal Integration of Wireless Technologies**: While both BLE and Internet technology each offer unique abilities, efficient integration of data paths and latencies from one domain to another was a challenge. Experienced computer architects and hardware engineers worked together over a period of three years to optimize the seamless transfer of data as it occurs between the two technologies while minimizing latency as much as possible.
13. **Customization of wireless protocols:** With minimal effort, researchers can define their stimulation time, parameters and characteristics to control an experiment in real time. With minimal programming knowledge, the data packets can be altered and redefined from both sending and receiving hardware of the IoTN ecosystem to help recreate and redesign communication protocols that are optimized for specific experimental needs.
14. **Global Collaboration:** With secure multiple laboratory access across the world, scientists can now easily collaborate with each other through this unified technology. This can be further extended to sharing of unused resources such as animals or compounds thus reducing possible wasted resources. Furthermore, this can potentially also provide opportunities to scientists in the developing world to remotely conduct experiments with their colleagues in the developed world.

The minimalistic and east-to-use setups integrated with customization of its various utilities highlight the real-world applications IoTN technology can provide when compared to contemporary commercial alternatives. This can not only offer opportunities to neuroscientists to conduct novel in vivo studies such as social behavior in a group or other similar complex experiments, but also provide applications for biomedical companies to control and customize large scale research to significantly reduce cost and time to market for new biotech products ranging from drugs to devices.

Our modification to the manuscript: The discussion section of the manuscript already includes discussion of our IoTN system, highlighting its uniqueness. Additionally, we added the following text along with a new table comparing our technology with other high-throughput systems (**Supplementary Table 1**) (p.20): "Compared to commercial high throughput systems, which only allow a locally accessed (minimal wireless control), limited application (minimal data type versatility and compatibility) and which is scalable to a limited number of multiplexed units, the IoTN ecosystem offers several unique advantages. The IoTN system provides a highly versatile and programmable universal wireless platform that can be globally accessed from anywhere and used with a diverse assortment of research laboratory equipment. Furthermore, this network ecosystem approach also allows multiplexed, real-time (or scheduled) and secure access to multiple remotely located laboratories around the globe. **Table 1** and **Supplementary Tables 1 and 2** detail the advantages and limitations of our approach compared to other relevant technologies."

- ***Comment #2:*** *Is this system truly scalable? The manuscripts claim that system can scale up to 7 animals in a local piconet and to hundreds overall. All their demonstrations, however, were with no more than a few animals. In addition, none of these demonstrations had high demand in data rate or fast control, which in many applications often are both required. In reality, Bluetooth and wifi both have limited overall bandwidth, leading to severe interference when device number goes up. (we all have experience with bad connections of Bluetooth mouse/keyboard) The authors should the capability of this system to handle large data stream, fast control and multiple devices, and report the true limits rather than theoretical limits.*

Our response: We appreciate the reviewer for the feedback. In order to properly demonstrate the higher scalability of our system, as suggested, we performed characterization experiments using 22 RCMs (Remote Control Modules) and *in vivo* neuroscience experiments using 21 RCMs (i.e., 21 mice), which were 3x more RCMs than the ones (= 7) used in our previous animal experiments. As shown in **Supplementary Figs. 6**, our Global Internet IoTN system offers good performance with low latency (~70 ms) and almost no failure (Success rate = 99.5%) when controlling 22 RCMs. In addition, we could successfully run simultaneous *in vivo* neuroscience experiments using 20+ mice, as demonstrated in **Fig. 5** and **Supplementary Fig. 8.**

However, as the reviewer mentioned, we agree that several issues, such as interference among RCMs and increased latency, can occur as the number of connected RCMs gradually increases, negatively affecting the result and accuracy of neuroscience experiments.

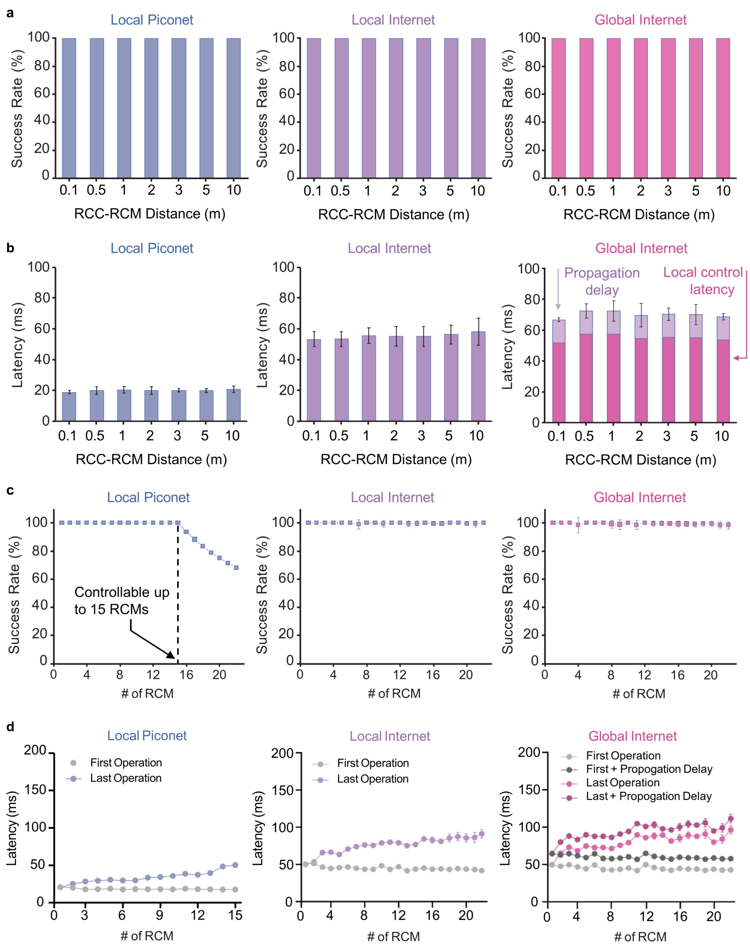
Indeed, Bluetooth technologies share the frequency bands of WiFi using a frequency hopping spread spectrum. While Bluetooth supports 79 designated Bluetooth channels to transmit data, BLE (Bluetooth Low Energy) uses 40 Bluetooth channels. Therefore, theoretically, the IoTN systems using BLE can support up to 40 simultaneous RCM controls. Since we found one BLE primary supports 4 to 15 BLE secondaries depending on the implementation, we designed our IoTN system to create multiple Piconets using multiple primary BLE devices. To scale up for tens to hundreds of RCMs, we also showed that our IoTN system could use Bluetooth Mesh technology. Also, the interference between WiFi and Bluetooth becomes less an issue since recent WiFi technologies use two additional frequency bands, such as 5 GHz and 6 GHz.

To further explain, conventional Bluetooth or BLE communication relies on direct communication between primary and secondary nodes. In particular, the primary node scans secondary nodes to establish connections to communicate. The secondary nodes can communicate with the primary node only through the already established connection. Although this primary-secondary communication is de facto standard and thus widely used, the primary node easily becomes the bottleneck in connection management. We observed that a primary BLE device could establish only up to 8 connections in practice (in the case of a single BLE dongle) even though the BLE bandwidth could allow 40 concurrent communication channels in a single network. However, our RCC (Remote Control Center) implementation overcomes this limitation by creating multiple Piconets, each of which is controlled by an individual primary BLE dongle, as stated in p.9.

While creating more Piconets by adding more BLE dongles could serve the purpose of small and mid-scale experiments, we also explored the Bluetooth Mesh technology for large scale experiments, scaling the network to hundreds of RCMs (**Supplementary Fig. 9**). Bluetooth Mesh builds a mesh network instead of a primary-secondary network, overcoming the BLE primary's bottleneck. In particular, it makes each node act as both a transmitter and a receiver using a managed flooding technique, which broadcasts the received messages to nearby nodes. This controlled flooding eliminates the bottleneck at the primary BLE nodes by letting the other BLE nodes in the network help deliver messages, thus reducing the total number of flooding messages. Our experiment in **Supplementary Video 5** showed the Bluetooth Mesh scales very well; 82 RCMs received a command successfully in the real field experiments. Theoretically, it can support 32,767 RCMs each with a unique address. Even though its practical limitation will be lower than the theoretical limit, we believe the Bluetooth Mesh technology's scalability is sufficient to support large scale neuroscience applications.

We aimed at controlling a large number of RCMs concurrently while exploiting energy-efficient BLE devices and Bluetooth mesh networks. For evaluation, we measured the successful message transmission rate and latency since they are most important for neuroscience applications. Since our optimized messages are 3-8 bytes long, measuring the aggregate throughput will not show any significant changes with more RCMs. We demonstrated the Bluetooth mesh network's performance controlled with our IoTN system in **Supplementary Video 5** and compared its concept with our current IoTN system with multiple Piconets in **Supplementary Fig. 9**. As demonstrated in our proof-of-principle experiment, scalability and reliability for the IoTN system can be significantly improved by integrating the Bluetooth mesh technology, which is a promising future solution.

Our modification to the manuscript: Supplementary Fig. 6 and its figure legend has been added as follows:



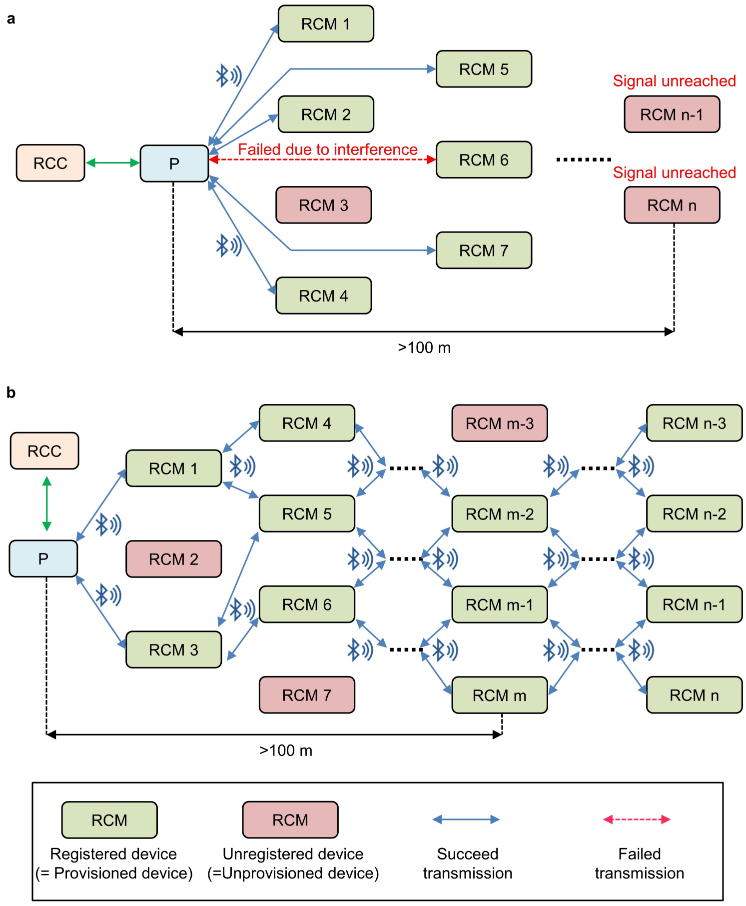
Supplementary Fig. 6 | Performance characteristics of local and global IoTN network. a-b, Success rate (**a**; n = 20) and latency (**b**; n = 5) of triggering μ-ILED neural probes with wireless RCMs as a function of different RCC-RCM distances (0.1, 0.5, 1, 2, 3, 5, and 10 m), comparing the responsiveness of local piconet, local internet, and global internet modes. The purple-color parts in the global internet latency graph (**b**, right) indicates a signal propagation delay, which is dependent on the inter-distance between the remote user and RCC (e.g., 15 ms delay between Boulder, CO and St. Louis, MO, USA). **c-d,** Success rate (**c**; n = 20) and latency (**d**; n = 5) of triggering a μ-ILED in optogenetic neural probe integrated with wireless RCMs as a function of different numbers of RCMs (1 - 22; 1 m distance from RCC), comparing the responsiveness of local piconet, local internet, and global internet modes. Note that the latency of the global internet control is determined by summing the local control latency and the distance-dependent propagation delay.

We also added the sentences as follows (p.16-17 & 24): "Scaling wireless networks can introduce issues of network crowding and interference. While these two discrete and independent behavioral experiments were successfully conducted twice within the same animal facility, this facility did not have excessive miscellaneous and unconnected Bluetooth devices within range of the minicomputer. At any given time, there were typically ~10 non-related devices representing BLE-enable security cameras, smart building infrastructure, smartphones, and computer peripherals. Increasing experimental scale to tens or hundreds of devices, however, could be limited due to signal inference among these and unconnected devices. One approach to limiting this interference would be to incorporate advanced Bluetooth mesh technology[34] into the IoTN ecosystem. In a proof-of-principle benchtop experiment, we demonstrate this integration by enabling selective device activation of 82 devices over a physical distance of 4 meters (**Supplementary Video 5** and **Supplementary Fig. 9**). This initial test suggests that advanced Bluetooth mesh technology is a feasible approach to limit Bluetooth signal interference in the IoTN ecosystem."

"Large-scale experiments with tens to hundreds of devices can be limited due to the Bluetooth signal inference among the devices. The potential of the interference can be eliminated by adopting an advanced Bluetooth mesh technology[34] into the current IoTN system, as shown in our proof-of-principle experiment (**Supplementary Video 5** and **Supplementary Fig. 9**)."

In addition, **Supplementary Video 5** and **Supplementary Fig. 9** have been added as follows:

Supplementary Video 5. Wireless control of multiple devices using Bluetooth mesh technology.



Supplementary Fig. 9 | Schematic diagram comparing the signal flow of the conventional Bluetooth communication and Bluetooth Mesh network. a, Block diagram showing one-to-many topology of the conventional Bluetooth network. A single piconet network (or RCC, in the case of Local Piconet) transmits signals to multiple registered RCMs. Failure in transmission can occur due to interference among RCMs (e.g., RCM 6), and RCMs located at a far distance (i.e., >100 m) from a piconet network are unable to be registered (e.g., RCMs n-1 and n). **b**, Block diagram showing many-to-many topology of Bluetooth Mesh network to highlight its reliability and scalability. Every node (i.e., RCC, piconet network, and registered RCMs) form a mesh network and act as both transmitter and receiver. By delivering the received signals to nearby registered RCMs, the number of RCMs and the distance between RCC and RCMs barely affect the performance of the system.

- ***Comment #3:*** *Were these experiments fully approved by the IACUC's in their institutions? These experiments involve researchers in a different institute/country to operate animal experiments and potentially broadcasting video streams of animal experiments if things go wrong. Were there special measures and trainings involved in these protocols?*

Our response: These animal experiments were fully approved by each institution's IACUC. Each behavioral experiment was conducted in accordance of IACUC approved protocols and managed by experimenters with years of behavioral assay experience. While our IoTN system enables fully-remote and global access, it is important to note that none of the experiments in this manuscript were performed without the necessary human oversight to ensure animal safety. Our laboratory is purposely set up with local remote (outside of the behavioral procedure rooms) video access so that experimenters can monitor animal behavior without direct interaction with the animals. These video feeds aid in designing careful experimental design, but also provide onsite investigators with vastly more information about animal welfare than is typical in most closed-box behavioral experiments. While all experimental modifications were monitored closely by personnel onsite, the environmental sensing can be safely achieved remotely and this study was conducted from ~350 km outside of the laboratory. However, because these studies take place in the homecage, animal welfare was ensured through daily animal husbandry health and safety checks as with any other homecage in our colony.

Our modification to the manuscript: None

- ***Comment #4:*** *1) Point 3 leads to the question regarding the overall security concerns of this strategy. Wifi, cellular network and Bluetooth are not sufficiently secure to guarantee the safety of the animal and the data.*
  2) Besides, there are many uncertainties involved here. For example, are there trained technician required within the piconet while the experiments were remotely controlled? If not, what if human intervention is required suddenly? (For example, animal escape or fighting)?
  What if a remote researcher initiated a potentially harmful stimulation/modulation, and his or her cell phone loses internet?
  What if researchers in country A operate animal experiments in country B, but did not fully follow the protocol and caused animal welfare issues? Who will be responsible?

Our response:
1) Our IoTN system requires user credentials for access, and thus only authorized users can access and conduct the experiment. The HTTP connection from a remote researcher to the IoTN system is encrypted using SSL (Secure Socket Layer), and so eavesdropping on the communication channel is not possible. Furthermore, the IoTN system can easily support IPSec (Internet Protocol Security), creating site-level security by connecting a remote network to a local network.

2) In every case, the users given access to any IoTN system with animal control were fully trained and approved to conduct animal research directly. As described above and in now in the manuscript itself, no animal studies were performed completely remotely as this was not necessary for demonstration of the technology. Remote operation was conducted to validated signal latencies and for phantom animal experiments used in the

supplementary videos. As with any animal research, all necessary safeguard for animal health and safety should be in place prior to and during implementation of IoTN-based experimentation. Particular legal and liability issues will be case dependent and should be established with contracts or memorandums of understandings between institutions prior to research conduct.

Our modification to the manuscript: We have added the following section to the discussion to directly address these points (p.22-23): "The incorporation of wireless networks into biomedical sciences carries numerous ethical considerations for the health, safety, and security of the research subjects involved. While our IoTN system enables fully-remote and global access, it is important to note that none of the experiments in this manuscript were performed without the necessary human oversight to ensure animal safety. While all optogenetic and pharmacological modifications were monitored closely by highly-trained, onsite personnel, the environmental sensing represents an opportunity to assess laboratory conditions remotely. This can be safely achieved remotely and this particular study was conducted from ~350 km outside of the laboratory (**Figs. 6f-i**). However, because these studies take place in the home cage, animal welfare was ensured through daily animal husbandry health and safety checks as with any other home cage in the animal colony. As researchers begin to incorporate wireless network ecosystems into their experimental workflows, it is absolutely necessary that investigators carefully vet the potential outcomes. If a neural manipulation is being operated remotely, appropriate safeguards need to be in place to mitigate any potential harm to the research subject. These considerations are not dissimilar to any home cage-based study such as those for circadian and feeding behaviors[33] where experiments often occur over many hours with the experimenter in a separate location.

The IoTN system requires user credentials for access, and thus only authorized users can access and conduct experiments. The connection between the users and the IoTN system's webserver is encrypted using HTTPS (Hypertext Transfer Protocol Secure), and so eavesdropping on the communication channel is not possible. Furthermore, the IoTN system can easily support IPSec (Internet Protocol Security), creating strong site-level security by connecting a remote network to a user's local network. The Bluetooth communication between an RCC and RCMs is not encrypted since they are usually located nearby (a few meters) inside the protected lab facilities. However, care should be taken prior to granting authorization to new users of the IoTN. In particular, the IoTN can likely reduce or streamline skilled labor involvement with some research studies. For example, **Fig. 5** and **Supplementary Fig. 8** were both conducted by single researcher – a feat that would have been difficult or impossible to do alone with the requisite temporal control. However, this should not mean that sophisticated and delicate animal experiments should be ported to less trained individuals. The use of a wireless control ecosystem must be restricted to those with the necessary experience and training to ensure animal safety. While one can imagine cooperative arrangements to conduct studies globally in a safe and secure manner, this should never be done without the necessary safeguards for animal health and well-being. On that note, in addition to potentially reducing skilled labor hours, the IoTN also has the potential to reduce and refine animal studies[43]. Enabling long-term home cage-based experiments should reduce the number of animals needed by increasing statistical power in within-subject designs. In this same vein, wireless home cage experiments, especially those that allow animals to be studied without human interaction, should help reduce stress to the animal subjects inherent to behavioral testing."

REVIEWER 2

- ***Summary Recommendation:*** *The manuscript "Scalable Wireless Network Ecosystems to Automate Behavioral and Circuit Neuroscience" describes the design and deployment of a multi-specimen optogenetic stimulation system. The Authors present an affordable and scalable probe that can be implanted in a large number of animals, and that can be controlled using wireless network infrastructure dubbed "Internet of Things in Neuroscience." (IoTN) The system allows to control up to 7 devices using a single smartphone and more with the user base minicomputer. The minicomputers can be theoretically connected over the Internet which would allow to run simultaneous experiments in multiple separate laboratories. The high throughput neuroscience experiments are demonstrated in the mouse and rat models. The presented experimental methods are very clear and thorough, which is another strong side of this manuscript.*

Described demonstration of high-throughput neuroscience experiments has significant importance and without a doubt will be stimulating to the field. Affordable, scalable and automated systems can enable new experimental designs that would otherwise be very troublesome for the human experimenter to oversee in person. However, even though the concept of IoTN is highly valuable, there is a certain overlap with the previous article by some of the Authors (Nature Biomedical Engineering volume 3, pages655–669(2019)) to substantiate publication of this manuscript in its current form. What can make this study stand out is the real demonstration of high-throughput experiment with 20+ animals and/or synchronized study between the animal groups in different laboratories/facilities/universities so that the Authors fully demonstrate the premise of IoTN.

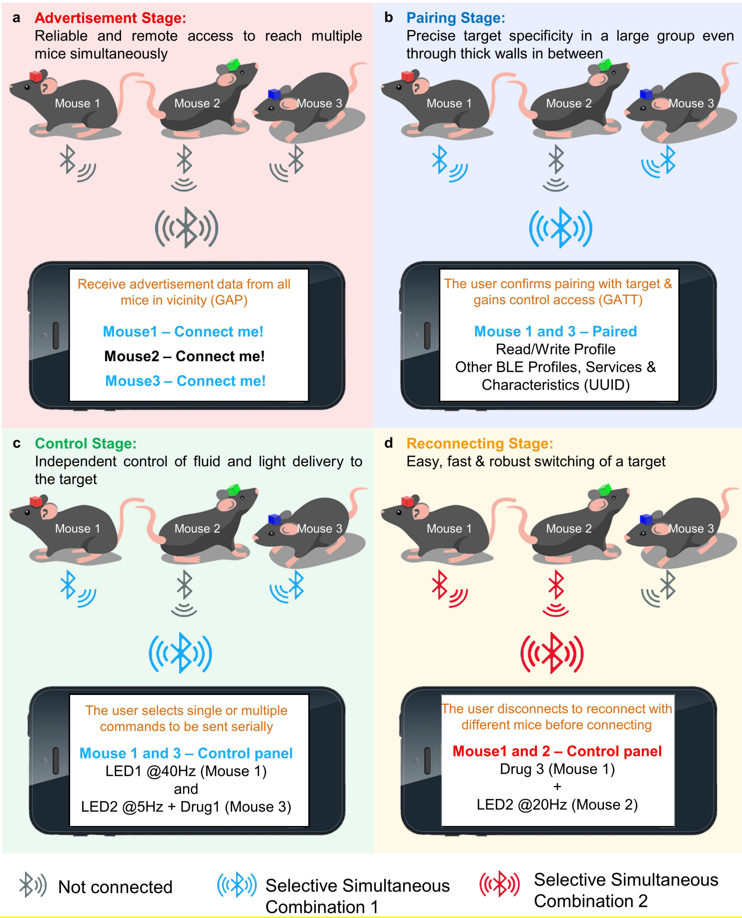
Our response: We appreciate the reviewer's positive comments and suggestion of new experiments for improvement. We have taken the reviewer's comments into consideration and modified our manuscript accordingly as detailed in our responses beneath respective comments.

- ***Comment #1:*** *While it is understandable that a lot of computer code can be reused, it should be very cautious about reusing figures. Supplementary Fig. 1 was conceptually very similar to the Supplementary Fig. 14 in Nature Biomedical Engineering, volume 3, pages 655–669 (2019). Supplementary Fig. 3 is Figure 3b in the previous article. The Authors should correct this and avoid similar issues in the future.*

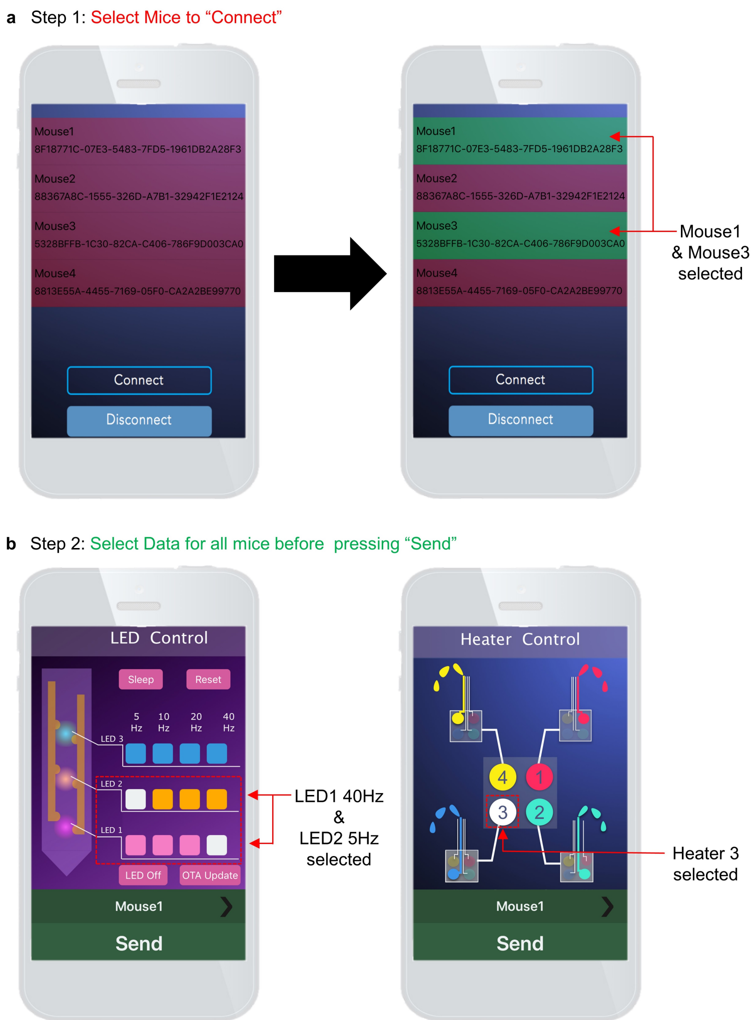
Our response: We appreciate the reviewer's comment, and we agree that **Supplementary Figs. 1** and **3** look similar to some of the figures of our research team's previous article (*Nature Biomedical Engineering* **3**, 655–669 (2019)). Following the reviewer's comment, we modified **Supplementary Figs. 1** and **3**.

Please note the following differences in these supplementary figures:
Supplementary Fig. 1 looks conceptually similar to Supplementary Fig. 14 in nBME (2019), but in fact, it is different in a sense that our local IoTN control based on the piconet is capable of "simultaneous selective" control, which is a clear advantage over the previous technology which allowed only one device control at a time.
Besides, although the graphical user interface of the smartphone app in **Supplementary Fig. 3** looks the same as the one shown in our previous publication (nBME (2019)), the internal software that allows simultaneous selective controls is new and different; with this new software design, we can select and control multiple devices "simultaneously and independently" as illustrated in **Supplementary Fig. 3**.

Our modification to the manuscript: Supplementary Figs. 1 and **3** have been modified as follows:



Supplementary Fig. 1 | Schematic illustration of selective simultaneous control in an IoTN network. **a**, Advertisement Stage: The advertisement information of all neural devices (red, green, blue) in the vicinity of the Remote Control Center (RCC) is collected by the RCC (smartphone or webserver), which is then displayed on the its graphical user interface (GUI). This information is handled by the Generic Access Profile (GAP) part of the Bluetooth Protocol Stack. Each mouse will have a unique advertisement name and such identification can help researcher select a specific group of mice within a large cohort with 100% accuracy. **b**, Pairing Stage: After the user selects a custom group of target mice (red and blue here) through the GUI of the RCC and clicks on the “Connect” button, all target devices stop advertising and enter into “connection mode” or “paired state”, while the other remaining devices keep advertising (available to other users in the IoTN network). The RCC then can access the various services and characteristics of the devices. This information is handled by the Generic Attribute Profile (GATT) of the Bluetooth Protocol Stack. **c**, Control Stage: After the target devices are paired, the user has the ability to selectively and simultaneously trigger one or multiple output modalities in each target device (independent of each other) within the paired subgroup. **d**, Reconnecting Stage: After finishing controlling a specific group of devices (red and blue), they can be easily disconnected and then will start advertising again. Meanwhile, a new subgroup of mice can be paired with the network, following the same communication protocol (steps **a** to **d**).



Supplementary Fig. 3 | Smartphone user interface for local piconet control, showing control of multimodal RCMs in two simple steps. **a**, Connect multiple target mice and/or IoTN enabled conventional equipment. **b**, Select multiple multimodal commands for each connected mice (by switching between devices using arrows on right and left of the device name) before finally pressing "Send" button. The smartphone app also has ability to provide timestamped confirmations which can be seen upon shaking the phone while the app is running.

- ***Comment #2:*** *For an article to be accessible to the broad audience, I think that the authors should look carefully for computer science terms that might be very new, niche or confusing. I believe that the word "Piconet" does not really exist in common scientific parlance and should be introduced and explained. The Authors could consult colleagues without expertise in computer science to identify other confusing terms.*

Our response: We thank the reviewer for the feedback. We used Piconet to represent a widely-used Bluetooth technology protocol that supports the primary-secondary type communication. More specifically, Piconet (Zaruba *et al.*, 2001) is an ad hoc network that connects the primary node and multiple secondary nodes using Bluetooth technology. It supports one primary node interconnects with one or more active secondary nodes (e.g., 7 active secondary nodes for Bluetooth and implementation-dependent for BLE). Also, the primary node can bring into the active status from up to 255 inactive secondary devices. For clarification, we added the definition of Piconet in our manuscript.

Our modification to the manuscript: We added the formal definition of Piconet on p.4 as follows: "The wireless IoTN network can either be controlled locally through BLE piconets (ad hoc network that connect the primary node and multiple secondary nodes using Bluetooth technology[21]) using a smartphone (**Fig. 1a**), or can be accessed globally over the Internet (**Fig. 1b**; see **Table 1** for detailed features and comparison to existing wireless technologies)."

- ***Comment #3:*** *Figures 1 and 2 feel very crowded, the Authors could try to reduce their complexity. Especially, in Fig. 2d(iii) I am unsure if all the peripheral elements of minicomputer are necessary on the scheme. Also, in Fig. 3c multiple abbreviations are used and "P" is never defined. Both of these figures could be made more condensed and clear.*

Our response: We agree that some parts of the **Fig. 2** may seem complicated to the readers, so we simplified **Figs. 2b** and **c**, following the reviewer's comment. Also, for **Fig. 2d(iii)**, we replaced the peripheral elements, except the BLE dongles, with an illustration of a Raspberry Pi, as we agree that they are unnecessary to be shown here**.** Instead, we moved the schematic of Raspberry Pi components to **Supplementary Fig. 2b**, as it may help the readers understand the structure of the minicomputer. We believe that the other parts of the figure are clear and straightforward, so we left them unchanged as they were. Regarding the abbreviations, RCC and RCM are clearly defined as 'Remote Control Center' and 'Remote Control Module', respectively, in the figure caption as well as in p.7 of our manuscript. "P" is also defined in the figure caption as "Piconet".

We feel that **Fig. 1** serves a purpose, in the sense that it visually conveys the ideas. We prefer to leave it as is.

Our modification to the manuscript: Figure 2, **Supplementary Fig. 2**, and their figure legends have been modified as follows:

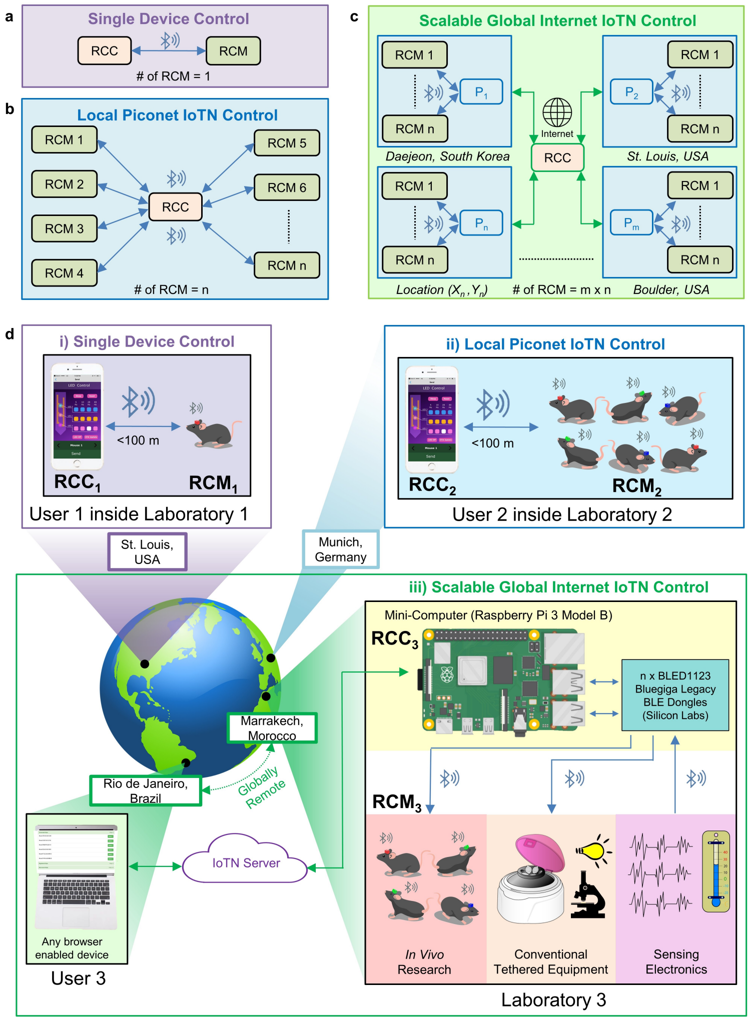
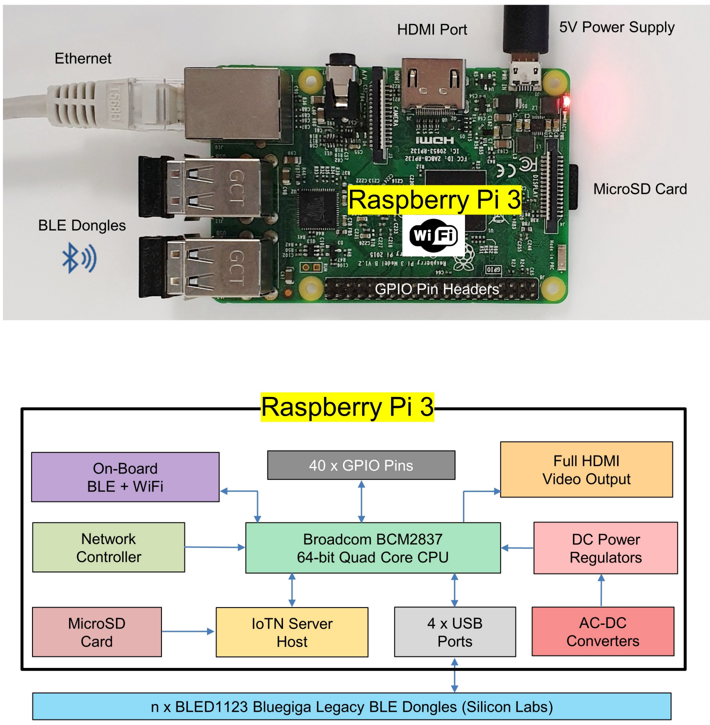


Fig. 2 | The IoTN network architecture and signal flow. **a-c**, Block diagram of network topologies to highlight topological differences and signal flow between (**a**) conventional Bluetooth single device control and (**b-c**) IoTN multi-device control modes, where RCC, RCM, and P stand for remote control center, remote control module, and piconet, respectively: (**b**) Local Piconet IoTN Control through a single RCC and (**c**) Global Internet IoTN Control through multiple piconet networks (P_1, P_2, P_3, …, and P_m), all of which are connected to a single RCC. **d**, Illustration of capabilities and access range of different technologies – i) Single device control where 1 RCC can control only 1 RCM at a time in its vicinity using BLE, ii) Local Piconet IoTN mode where a single RCC can selectively and simultaneously control up to 15 independent RCMs within its vicinity using BLE and iii) Global Internet IoTN mode where a single remotely located RCC can selectively and simultaneously control multiple devices across the globe at a different laboratories (equipped with a minicomputer loaded with a custom IoTN operating system) using combination of internet and BLE technologies. The IoTN technology can control not only multimodal neural implants, but also tethered conventional equipment while gathering data from multiple analog and digital sensors at the same time.

a

b



Supplementary Fig. 2 | IoTN minicomputer setup for global control mode. a-b, Optical image (**a**) and schematic component diagram (**b**) of a minicomputer (Raspberry Pi 3 Model B). To enable global IoTN control mode, a minicomputer is used to host a custom IoTN webserver (loaded inside its microSD card) over the Internet. Furthermore, multiple BLE dongles (BLED112, Silicon Labs) allow it to communicate seamlessly with numerous wireless RCMs in the vicinity (<100 m) once the command is received and decoded from the remote user. 40 GPIO pins enable access to the external equipment while the input power supply helps power various electronic components on the minicomputer. The ethernet port helps provide fast internet access. The HDMI port can be used to debug the minicomputer.

- ***Comment #4:** How many devices can be connected per minicomputer? Is it only 7 or 7 per BLE dongle?*

Our response: We apologize for the lack of clarification about the information. A maximum of eight devices can be connected to a single BLE dongle (BLED112, Silicon Labs), which means that the user can connect as many devices as eight times the number of BLE dongles connected to the minicomputer. Concerning that, it may give some misunderstanding to some readers, we included the additional explanation in the Results section.

Our modification to the manuscript: We added a sentence as follows (p.9): "By increasing the number of BLE dongles in the minicomputer, the synchronized hopping and time division multiplexing techniques can be parallelized such that each individual piconet sub-network can wirelessly, selectively and simultaneously control numerous devices at a time. The number of controllable devices scales by increasing the number of connected BLE dongles. Each individual BLE dongle can support up to eight connections. Our custom software driver quickly adapts data multiplexing algorithms and wireless data streaming based on the number of dongles connected for efficient and reliable wireless communication to all end devices."

- ***Comment #5:*** *I find the sentence on p. 7: "We used this identification technique to enable Local Piconet mode to control devices simultaneously and selectively with 100% accuracy. little misleading because with 48-bit Bluetooth address space the chance of incorrect pairing is close to 0%. The Authors could clarify that this is a comparison to IR- or RF-controlled devices which do not offer digital fidelity of the Bluetooth technology.*

Our response: We thank the reviewer for pointing this seemingly subtle critique. We modified the text following the reviewer's suggestion.

Our modification to the manuscript: We modified the sentence as follows (p.7-8): "We used this identification technique to enable Local Piconet mode to control devices simultaneously and selectively with 100% accuracy (**Supplementary Fig. 6**), in comparison to contemporary technologies such as RF[8–11,13,22,23] or IR technologies[7,24], which do not have selective capabilities leading to potentially limitless inaccurate pairings."

REVIEWER 3

- ***Summary Recommendation:*** *The authors report a novel wireless method to conduct neurophysiological experiments. The stated intent of the report is that this capability will allow one to remotely and independently control multiple behavioral/neurophysiological experiments simultaneously in rodents (or other animal models) involving optogenetic stimulation, drug delivery, electrical stimulation, sensing basic environmental conditions such as temperature, pressure, humidity or perhaps neurosensing (although no examples or data are suggested) etc. The authors propose a local network called a "piconet" using conventional Bluetooth low energy (BLE) technology which allows the control of multiple independent experiments within 100 m (range of Bluetooth systems). In addition, the authors propose a novel IoTN (Internet of Things in Neuroscience) method to integrate multiple piconets anywhere in the world through a cloud approach. Therefore, the IoTN can be used to control BLE through a cellular phone or access the internet through Wi-fi or the cellular network and access internationally located piconets.*

Summary of results - The local BLE will be useful in simultaneously controlling behavioral experiments involving a maximum of 7 animals using a local user. While the Wi-fi capability and webserver running on a local Raspberry PI minicomputer will allow the user to receive data from multiple locations around the world. So, in terms of hardware requirements, one needs only a mobile phone for local piconet setup (for a BLE) and an additional Raspberry PI minicomputer for worldwide access through Wi-fi. The devices (remote control modules or RCMs) will need a radio receiver or transceiver and a 48-bit unique address to communicate with the central RCC (remote control center) node within each piconet. The authors describe two modes of operation – the local piconet mode (or P mode) for local operation of the piconet and the global internet (or I mode) for accessing a remote cloud network that enables control and access around the world.

1.To demonstrate effectiveness, the authors use the local piconet and global internet to control the feeding behavior of AgRP expressing mice by stimulating the PVH (paraventricular nucleus of the hypothalamus) using micro-LEDs.

2.Simultaneous and selective control of multiple experiments through IoTN. Micro-LEDs were implanted in the motor cortex of 6 mice and were activated through local piconet to increase their locomotor activity.

3.The authors demonstrate selectivity through activation of 2 distinct opsins (inhibitory and excitatory) through optical stimulation of implanted micro-LEDs in the VTA of mice. Using amber and blue light they were able to either increase social interaction in a statistically significant way or decrease social interaction (not statistically significant).

4.To demonstrate the versatility of the IoTN, the authors demonstrate the ability to activate a microfluidic device to pharmacologically manipulate the food consumption of transgenic mice.

5.Finally, to demonstrate the ability to perform delayed scheduling of IoTN activated experiments (thereby eliminating the need for an experimenter to be present and arguably eliminating the 'observer effects'), the authors schedule the activation of the arousal modulating properties of the locus coeruleus noradrenergic system using an fiber optic excitation of the pre-frontal cortex.

Innovation – BLE technology exists already and there is nothing in this report that appears to add to that capability. BLE in its current form can also operate in mesh configurations

to significantly scale up the number of different experiments that can be controlled. The innovation in this report appears to be two-fold:
a.Addition of a capability to wirelessly access a cloud network through Wi-fi and webserver on a local minicomputer.
b.Characterization of the resultant IoTN system in 5 distinct neurobehavioral experiments, 4 of which involved micro-LEDs or optic fibers and one involved a microfluidic device.

Degree of advance - In my opinion, this is a methodology report. The degree of advance in the methodology proposed by the authors is incremental to the existing state-of-the-art in wireless communications.

Implications - The demonstrations in this study are too specific and too early. For a report on a new method, it has to be much more focused on the technology with a far greater rigor in design and testing than on the applications as this study does.

Our response: We thank the reviewer for the comment. At first glance, the IoTN technology might seem incremental and a straightforward integration of off-the-shelf components and protocols with applications comparable to some commercial behavior setups used in neuroscience laboratories. However, instead of "a remotely controlled software with local off-the-shelf hardware", the IoTN technology is in real-sense a wholistic customizable wireless ecosystem with the following novel values:

a. **Minimalistic hardware setups with unique wireless neural devices:** This not only includes off-the-shelf minicomputers, Bluetooth dongles and conventional equipment but also our untethered, modular, rechargeable and wirelessly programmable IoTN control modules as well as soft, bio-compliant and multimodal neural probes, which together offer opportunities for untethered, chronic *in vivo* studies such as wireless optogenetics, drug delivery or optopharmacology. Our wireless IoTN control modules can be easily swapped with different types and sizes of neural probes as well as different animals and/or other commercial equipment, thus enhancing overall versatility to neuroscientists with limited resources.
b. **Customizable and optimized software:** The software infrastructure here not only includes a globally accessible website and a custom-designed smartphone app, but also our custom-defined communication architecture and driver infrastructure for customizable data packets, real-time data multiplexing and adaptable data streaming enabling unmatched scalability to a large number of devices. The protocols, data structures and communication infrastructure we separately engineered and continuously optimized over a period of three years for their optimal integration, maximum reliability (nearly 100% success rate), minimal latency (~20 ms for the piconet and (~55 + α) ms for the internet control; α = global signal propagation delay) and seamless functionality together as a multi-wireless system. Note that the propagation delay (α) can be completely eliminated and ignored if using the scheduled control, where RCC uses only the local network.
c. **Novel applications for *in vivo* studies:** By optimally integrating the versatility and modular construction of both unique IoTN hardware and software within a single ecosystem, the IoTN technology is able to complete unique applications which have not been demonstrated before by a single standalone technology. This includes, but is not limited to low cost (~$45) and minimalistic hardware, global remote access, unmatched selectivity and scheduling (both at device level and at multimodal output level), customizable automation and high-throughput scalability. Upon its mass deployment, there can be new avenues for global collaboration within the scientific community, especially in developing countries that lack significant infrastructure and funding to carry out complex or large-scale studies.

To properly design and demonstrate the capability of the IoTN technology, the design requirements had to be derived from the applications that this was built to address and for applications that would be commonplace for potential future users. Since we are not developing a generic IoT system, but a very specific system focused on behavioral and circuit neuroscience applications (which has not been integrated with such technology before), it became important that we base IoTN technology design requirements on various real-life neuroscience applications. Therefore we present our research to the community as a balance between the design technology (that appeals to the engineering community) and its various real-life neuroscience applications (that appeals to the neuroscience community) to highlight both the engineering aspects and the reliable *in vivo* functionality that neuroscientists can readily employ for their *in vivo* research. Furthermore, feedback from the reviewers helped us perform additional engineering characterization of our technology including latency, error rates, the number of controllable devices, control modes, as well as push the scalability of *in vivo* demonstrations all of which helped further improve the manuscript.

We also refer Reviewer 3 to the 14 points made in response to Reviewer 1 regarding the unique advantages of the IoTN system we have designed and presented here.

Our modification to the manuscript: The discussion section of the manuscript already includes discussion of our IoTN system, highlighting its uniqueness. Additionally, we added the following text along with a new table comparing our technology with other high-throughput systems (**Supplementary Table 1**) (p.20): "Compared to commercial high throughput systems, which only allow a locally accessed (minimal wireless control), limited application (minimal data type versatility and compatibility) and which is scalable to a limited number of multiplexed units, the IoTN ecosystem offers several unique advantages. The IoTN system provides a highly versatile and programmable universal wireless platform that can be globally accessed from anywhere and used with a diverse assortment of research laboratory equipment. Furthermore, this network ecosystem approach also allows multiplexed, real-time (or scheduled) and secure access to multiple remotely located laboratories around the globe. **Table 1** and **Supplementary Tables 1 and 2** detail the advantages and limitations of our approach compared to other relevant technologies."

Major technical criticisms/weaknesses:

- ***Comment #1:*** *The weakness of this study is its design and the lack of focus. It is essentially reporting the development of a new capability or tool, which the authors refer to as the IoTN. The authors argue for the need for such a tool in neurobehavioral experiments where there is a need to control multiple experiments remotely all over the world. Technology development reports such as these should clearly lay out the different metrics of performance and the quantitative requirements for each of these metrics that the proposed technology should satisfy. The system design should then be motivated by these requirements. However, in this report, the required metrics of performance and the corresponding quantitative requirements are not explicitly laid out. The whole design process has an ad-hoc feel to it. The experiments to test and demonstrate the capabilities of the technology are needlessly complicated. Simple bench-top experiments could have provided much of the results that the authors are looking for, with far greater rigor.*

Our response: We thank the reviewer for the constructive feedback. First of all, for clarification, we summarize the need for the high throughput wireless neurobehavioral system, which we referred to as IoTN:

Neuroscience Requirements and the needs for the IoTN system

Consistent experiment control with a large number of animals: Conducting neuroscience experiments depends heavily on human resources, such as experienced experimenters, as these experiments often require complex yet sensitive controls on the subjects. Furthermore, involving a large number of experimental subjects with precise control is exceptionally challenging leading to many behavioral and circuit neuroscience studies being conducted in piecemeal fashion sometimes by running a single animal at a time for a study that will require at least 10s of animals to achieve the statistical powers required for interpretation. Wireless network systems can help support automated, real-time control for multiple subjects to enable higher throughput.

Repeatable and reproducible experiments: Scientific research must be reproducible. An automated system that controls many experimental subjects concurrently helps standardize experimental conditions to rigorously reproduce the same results. Wireless network systems support untethered communication to many subjects simultaneously or in real-time, enabling reproducible experiments. The sensing and schedule functions can also help ensure that timepoints and environmental conditions are repeated and standardized across replications.

Overcome geographical limitation: Researchers or experimentalists usually need to be on-site to conduct neuroscience experiments. The internet-enabled system that can securely communicate and run experiments can remove this geographical limitation.

Energy-efficient wireless network protocols: Because sensors or devices used in neuroscience applications are small and portable to be attached to animals, limited battery capacity is a significant constraint. Wireless network technologies such as Wi-Fi used for high-speed data transmission are not an ideal candidate for these neuroscience applications. We use the BLE to minimize energy consumption while satisfying application requirements such as real-time control and feedback from multiple sensors.

Scalability with many communication devices: Energy-efficient wireless network technologies such as BLE have limited bandwidth and communication ranges. For example, most BLE implementations only support 4-15 concurrent connections. We implemented RCC to coordinate multiple BLE dongles to create multiple Piconets at the application-level process to support an increasing number of RCMs.

Readily available and cost-effective: For broader adoption, the technology needs to be cost-effective and widely accessible. Our system supports a range of control and monitoring devices such as smartphones and Raspberry PIs. It can include any BLE devices attached to experimental subjects for control and monitoring using Bluetooth family protocols (BLE and Bluetooth Mesh).

As per the reviewer suggestions, we have performed several additional benchtop tests to help characterize our IoTN technology even further. Below we defined various key metrics of performance for our IoTN technology including latency, the number of controllable devices, and error rates, and discuss how our IoTN system meets the requirements:

1. **Latency**: For time-locked control of neural stimulation in behaviors that function on the scale of seconds, the latency should be at least an order of magnitude lower (i.e. less than 100 ms) on average to avoid significant delay for large scale or simultaneous experiments. Based on our latest tests, we were able to achieve ~20 ms and (~55 + α) ms for piconet and internet control modes, respectively, where α represents a signal

propagation delay between the remote user and RCC (**Fig. 3b** and **Supplementary Fig. 6b**). For closed-loop neural experiments, latency would necessarily need to be much lower (~1 ms) – closed-loop neural experiments that require a response to a single action potential necessitate a hardwired closed loop, but many other neuroscience studies can function without issue on the hundred of millisecond timescale. The majority of behavioral studies use video framerates that refresh every 30-200 ms, well within the range that our systems can respond to events. Even the sampling rate of calcium imaging of neural activity is often slower than the latencies we present here.

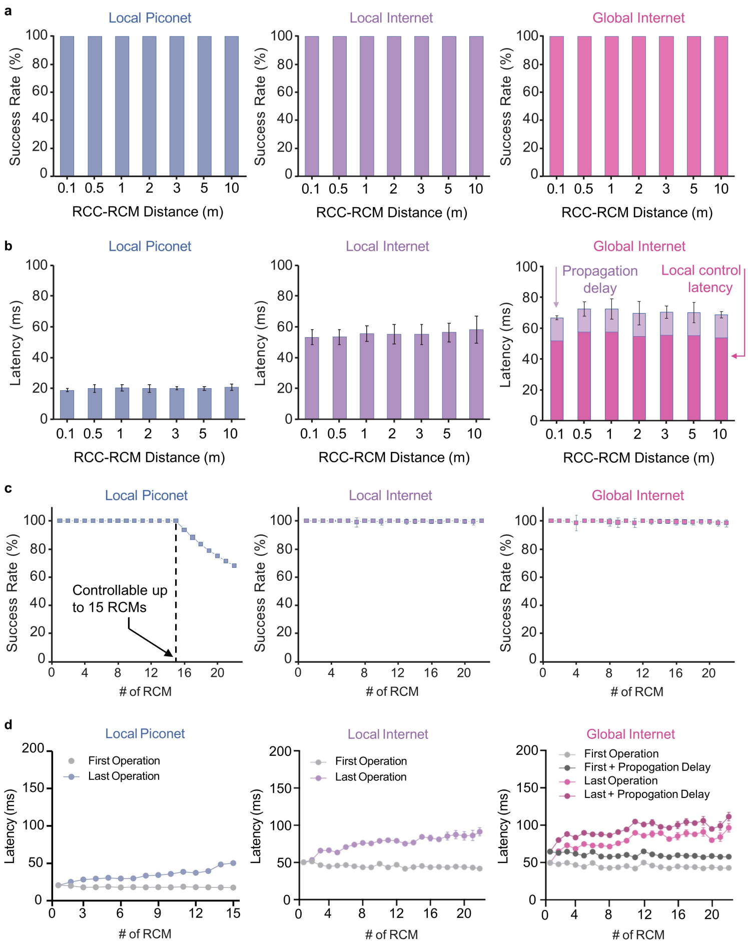
2. **The Number of controllable devices**: An ideal IoTN technology needs to be scalable to hundreds of devices to enable high-throughput experiments. Currently, for *in vivo* studies, we were able to demonstrate simultaneous control of 21 animals (using conventional BLE) and 82 benchtop devices using Bluetooth Mesh technology (**Supplementary Video 5**), which could be extended to 100+ devices (we were limited by hardware and animal availability during the COVID-19 pandemic).
3. **Error rates**: The IoTN should work reliably with nearly 0% error rate, regardless of whether the user uses a smartphone (locally over piconet) or from a different part of the globe (over the internet). Based on our recent tests, we found that the error rates for piconet and internet modes were both nearly zero (i.e., nearly 100% of success rate) once the RCMs were paired with an RCC (**Supplementary Figs. 6a** and **c**).

As discussed above, the performance metrics of our system satisfy the established requirements for IoTN technology. The below table summarizes the key required metrics of performance and the corresponding actual performance of our IoTN system:

IoTN Performance Metrics	**Requirement**	**Our IoTN System Performance**
Latency	<100 ms	~20 ms (piconet) / (~55 + α) ms (internet); α = propagation delay
The Number of controllable devices	Scalable to hundreds	15 (piconet) / 22+ (internet) / 100+ (Bluetooth Mesh)
Error rates	0%	~0%

Our modification to the manuscript: We modified the sentences as follows (p.11-12): “**Figures 3b** and **c**, **Supplementary Fig. 6**, and **Supplementary Videos 1-3** demonstrate the promptness and reliability of local piconet, local and global Internet control modes for remote control of these wireless optogenetic devices (see **Table 2** for summarized key performance metrics). Operation in all control modes is highly stable when a single RCM is paired to RCC, regardless of the distance between RCC and RCM (**Supplementary Fig. 6a**). These tests had zero failures (i.e., 100% of success rate). Transmission is faster in the local piconet compared to the Internet controls (latency ~20 ms for the piconet control vs. (~55 + α) ms for the Internet control where α represents a signal propagation delay between the remote user and RCC; **Fig. 3b** and **Supplementary Fig. 6b**). This increased latency for internet control is mainly due to increased RCC-RCM distance, making the local piconet control more suitable for sophisticated experiments requiring higher temporal control resolution. For scheduled controls, RCC uses only the local network, thus the signal propagation delay can be completely eliminated. Therefore, using the scheduled control can make the latency difference between local and global internet controls negligible. For simultaneous output or device control, the latency of additional devices receiving data increases since the frequency hopping algorithms need to find available frequencies for the additional devices (**Fig. 3c**). Overall, however, the differences are small and, in most cases, inconsequential. This is especially true for behavioral experiments with the time course of seconds or longer. Furthermore, the number of paired RCMs does not significantly affect the latency or success rate of BLE signal transmission (**Supplementary Figs. 6c** and **d**) except in the case of the local piconet where the number of allowable connections is limited to 15 by a smartphone. These results demonstrate the functionality and scalability of the IoTN control in reliably controlling numerous devices on millisecond timescales."

Also, **Supplementary Fig. 6** and its figure legend has been added as follows:



Supplementary Fig. 6 | Performance characteristics of local and global IoTN network. a-b, Success rate (**a**; n = 20) and latency (**b**; n = 5) of triggering μ-ILED neural probes with wireless RCMs as a function of different RCC-RCM distances (0.1, 0.5, 1, 2, 3, 5, and 10 m), comparing the responsiveness of local piconet, local internet, and global internet modes. The purple-color parts in the global internet latency graph (**b**, right) indicates a signal propagation delay, which is dependent on the inter-distance between the remote user and RCC (e.g., 15 ms delay between Boulder, CO and St. Louis, MO, USA). **c-d,** Success rate (**c**; n = 20) and latency (**d**; n = 5) of triggering a μ-ILED in optogenetic neural probe integrated with wireless RCMs as a function of different numbers of RCMs (1 - 22; 1 m distance from RCC), comparing the responsiveness of local piconet, local internet, and global internet modes. Note that the latency of the global internet control is determined by summing the local control latency and the distance-dependent propagation delay.

Comment #2: *The authors test and demonstrate this technology in 5 complicated neurobehavioral experiments with the unstated performance criteria such as – "effectiveness", simultaneous control, selectivity, "flexibility", ability to perform delayed scheduling of experiments. To develop a wireless technology to remotely sense and control neurobehavioral experiments (although no "sensing" was demonstrated in this study), there are several other performance metrics that are far more important and should be addressed carefully but are not addressed here such as - reliability, error rates, bandwidth issues in bidirectional communication, power consumption etc.*

Our response: We appreciate the feedback. First of all, we would like note that bidirectional sensing capability was actually demonstrated in our paper where we sensed temperature, pressure and humidity levels in the experimental home cages using the IoTN hardware and software. We believe that with further advancements in IoT communication technologies, a high bandwidth full-duplex stimulation (such as optogenetic and pharmacological) and neural activity monitoring (such as electrophysiological recordings and wireless calcium imaging) should be possible in the near future. We also agree that further characterization of various IoTN capabilities was needed to better highlight the technical properties of the IoTN system. Upon further testing, we characterized various performance metrics as suggested by the reviewer:

1. **Error rate vs Distance** (**Supplementary Fig. 6a**): Once a single RCM is paired with RCC, the IoTN system showed a 100% of success rate regardless of the distance between RCC and RCM.
2. **Latency vs Distance** (**Supplementary Fig. 6b**): The distance between RCC and RCM barely affects the latency of triggering the devices in both piconet and internet. Note that the latency in the global internet control increases due to a signal propagation delay between the remote user and RCC, but this delay can be eliminated by implementing the 'scheduled control', which uses a local network at pre-defined times.
3. **Error rate vs the Number of devices** (**Supplementary Fig. 6c**): Error rate of internet IoTN control network shows almost no failure (i.e., nearly 100% of success rate) regardless of the number of connected devices. On the other hand, while piconet IoTN mode shows perfect reliability (i.e., exactly 100% of success rate) when equal to or less than 15 RCMs are connected, the excess numbers of RCMs fail in operation when more than 15 RCMs are connected.
4. **Latency vs the Number of devices** (**Supplementary Fig. 6d**): The latency of the first operated device is almost the same regardless of the number of connected devices in both piconet and internet IoTN modes. In piconet IoTN mode, the "smartphone app" sequentially transmits the signals to the devices, which results in gradually increased latencies among the connected devices. On the other hand, in local network and internet IoTN modes, latency signals are sequentially transmitted from multiple "BLE dongles" to the connected RCMs, resulting in the variabilities of latencies among RCMs connected to the same BLE dongle. Due to these sequentially transmitted signals, in general, the latency to operate all the connected devices increases as the number increases. At a large scale, the effect of latencies can be partly mitigated by utilizing Bluetooth mesh networks where data can be transmitted through multiple pathways simultaneously in a large network.
5. **Latency vs Local/Global Internet control modes** (**Figs. 3b** and **c** and **Supplementary Figs. 6b** and **d**): the latency in the global internet control mode is

larger than the local internet control mode due to a signal propagation delay between the remote user and RCC. However, this delay can be completely removed by implementing the 'scheduled control' mode, which solely uses a local network at pre-defined times and therefore capitalizes on its reduced latencies.

6. **Power consumption**: Since most of the IoTN hardware is powered through wall-sockets except the wireless RCMs, we characterized the power for the wireless RCMs only. We found out that the power consumed by the wireless RCM at average was 39.6 mW when the μ-ILEDs are operated at 20 Hz frequency with a 10 ms pulse width. Hence the battery used in wireless IoTN RCMs was selected to be 12 mAh for use in mice brains to allow over 60 minutes of uninterrupted wireless operation. The battery requirement of wireless RCM can be varied based on the length of the required experiment and the size of the animal.

Our modification to the manuscript: We made modifications as written in our response to the reviewer's comment #1. In addition, we added **Table 2** that summarizes the performance metrics as follows:

Table 2. Performance metrics of IoTN system

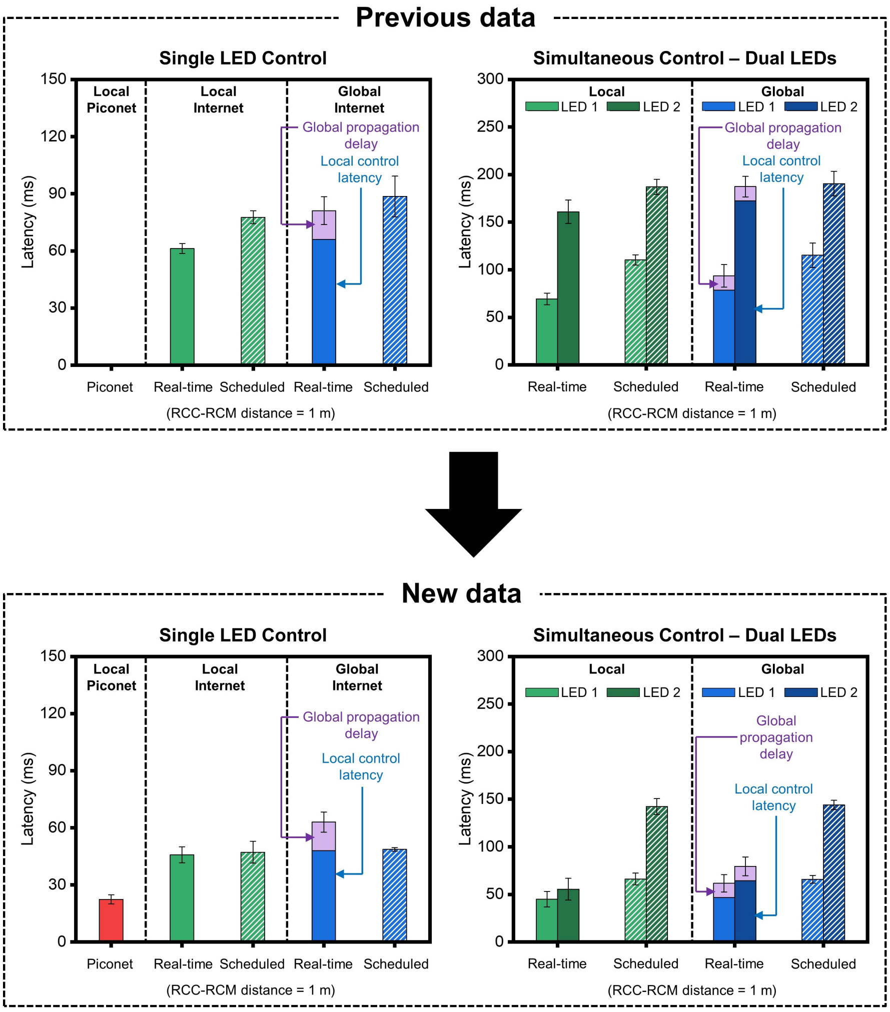
IoTN Performance Metrics	Local Piconet mode	Local Interent mode	Global Internet mode
Latency	~20 ms (max ~50 ms with 15 devices)	~55 ms (max ~90 ms with 22 devices)	(~55 + α) ms; where α = propagation delay (max ~ (95 + α) ms with 22 devices)
Error rate (= Misses/Trials x 100)	0% (with ≤15 devices)	<0.2% (regardless of number of devices)	<0.5% (regardless of number of devices)
Control distance between user and RCM	<100 m	No Limit	No Limit
The Number of controllable devices	15	22+ (100+ with Bluetooth Mesh)	22+ (100+ with Bluetooth Mesh)
Power consumption of wireless module	Tens of mW for μ-ILED operation	Tens of mW for μ-ILED operation	Tens of mW for μ-ILED operation
Bandwidth	~1 Mb/s (Bluetooth Low Energy, extendable up to 2 Mb/s for Bluetooth 5)	~1 Mb/s (Bluetooth Low Energy, extendable up to 2 Mb/s for Bluetooth 5)	~1 Mb/s (Bluetooth Low Energy, extendable up to 2 Mb/s for Bluetooth 5)

Also, we added a sentence as follows (p.11): "**Figures 3b** and **c**, **Supplementary Fig. 6**, and **Supplementary Videos 1-3** demonstrate the promptness and reliability of local piconet, local and global Internet control modes for remote control of these wireless optogenetic devices (see **Table 2** for summarized key performance metrics)."

Minor technical criticisms:

- ***Comment #3:** Reliability of IoTN – The authors use the piconet and global internet to control implanted micro-LEDs bilaterally in rodents. The authors report in Fig. 3b, a 0.75 ms latency using the local piconet and a 100 ms latency using the global internet. What was the RCM-RCC distance for this 0.75 ms latency and how does the latency, and error rate vary with RCC-RCM distance and the number of RCMs on the piconet? Also, there are several questions and concerns regarding the variabilities in the latencies with the global internet. Are these variabilities within each RCM-RCC pair over a period of time or are these variabilities across multiple RCMs?*

Our response: We appreciate the reviewer's constructive comment. Latencies shown in the original **Figs. 3b** and **c** were measured when the RCC-RCM distance was 1 m. After the initial submission of our manuscript, we modified our local piconet smartphone app due to the compatibility issue with an updated iOS version of the smartphone, which resulted in increased latency (i.e., increased from 0.5 ms to 20 ms) and the increased number of allowable connections (i.e., increased from 7 to 15). Though the latency of local piconet is increased compared to the previous version, 20 ms is still a sufficiently short to be used for most closed-loop neuroscience experiments as the majority of behavioral studies use video framerates that refresh every 30-200 ms. That being said, for neurophysiological studies that require extremely short latency (~1 ms) to achieve collision tests or neural activity based closed-loops, other technology may be required. On the other hand, more than a doubled number of allowable connections enables higher throughput in local animal experiments. For better reliability, we also improved the performance of the IoTN system.
After the modification in both piconet and internet IoTN systems, we retested their characteristics, including the latency and success rate (= 100% - error rate) depending on the RCC-RCM distance and the number of paired RCMs. The below graphs (**Figs. 3b** and **c**) compare the previous and updated latencies of the IoTN system in a local piconet, local internet, and global internet modes. Note that for the global internet latency, a signal propagation delay between RCC and the remote user needs to be considered and added. This value is dependent on the inter-distance between the remote user and RCC. For examples, there is 70 ms delay between Boulder, CO, USA and Daejeon, South Korea, and 15 ms delay occurs between Boulder, CO and St. Louis, MO, USA.
We also updated **Supplementary Video 3** (Global Internet remote control using IoTN technology) using an improved website.



Updated results shown in **Figs. 3b** and **c** and **Supplementary Fig. 6** clearly indicate the reduced latency and nearly flawless success rate of the internet IoTN network, which are not significantly varied by RCC-RCM distance or the number of paired RCMs. Piconet control shows short latency (~20 ms) and perfectly stable operation without any error (i.e., success rate = 100%) regardless of the RCC-RCM distance, but the number of pairable RCMs does not exceed 15 (**Supplementary Figs. 6c**, left), which shows the limitation of piconet in scalability-required experiments. In contrast, although the latency is slightly increased compared to piconet control (~55 ms for local internet control and (~55 + α) ms for global internet control, where α represents a signal propagation delay between the remote user and RCC), reliable operation with allowable latency is shown regardless of the number of paired RCMs (**Supplementary Figs. 6c** and **d**, middle and right).
Variabilities of latencies in the local and global control occur within a BLE dongle-RCM pair. A single BLE dongle cannot simultaneously transmit the BLE signals to connected RCMs (which the maximum number is 8 per BLE dongle). Instead, the signals are "sequentially" transmitted from a BLE dongle to the connected RCMs, which results in the variabilities of latencies among RCMs, which are connected to the same BLE dongle. In order to minimize the latency, the IoTN website was designed to pair the new RCM with a BLE dongle with the least number of currently connected RCMs, so that the number of RCMs connected to each BLE dongles can be minimized.

Our modification to the manuscript: We modified the sentences as follows (p.11-12): "**Figures 3b** and **c**, **Supplementary Fig. 6**, and **Supplementary Videos 1-3**

demonstrate the promptness and reliability of local piconet, local and global Internet control modes for remote control of these wireless optogenetic devices (see **Table 2** for summarized key performance metrics). Operation in all control modes is highly stable when a single RCM is paired to RCC, regardless of the distance between RCC and RCM (**Supplementary Fig. 6a**). These tests had zero failures (i.e., 100% of success rate). Transmission is faster in the local piconet compared to the Internet controls (latency ~20 ms for the piconet control vs. (~55 + α) ms for the Internet control where α represents a signal propagation delay between the remote user and RCC; **Fig. 3b** and **Supplementary Fig. 6b**). This increased latency for internet control is mainly due to increased RCC-RCM distance, making the local piconet control more suitable for sophisticated experiments requiring higher temporal control resolution. For scheduled controls, RCC uses only the local network, thus the signal propagation delay can be completely eliminated. Therefore, using the scheduled control can make the latency difference between local and global internet controls negligible. For simultaneous output or device control, the latency of additional devices receiving data increases since the frequency hopping algorithms need to find available frequencies for the additional devices (**Fig. 3c**). Overall, however, the differences are small and, in most cases, inconsequential. This is especially true for behavioral experiments with the time course of seconds or longer. Furthermore, the number of paired RCMs does not significantly affect the latency or success rate of BLE signal transmission (**Supplementary Figs. 6c** and **d**) except in the case of the local piconet where the number of allowable connections is limited to 15 by a smartphone. These results demonstrate the functionality and scalability of the IoTN control in reliably controlling numerous devices on millisecond timescales."

Also, **Figures 3b and c** and their figure legends have been modified as follows:

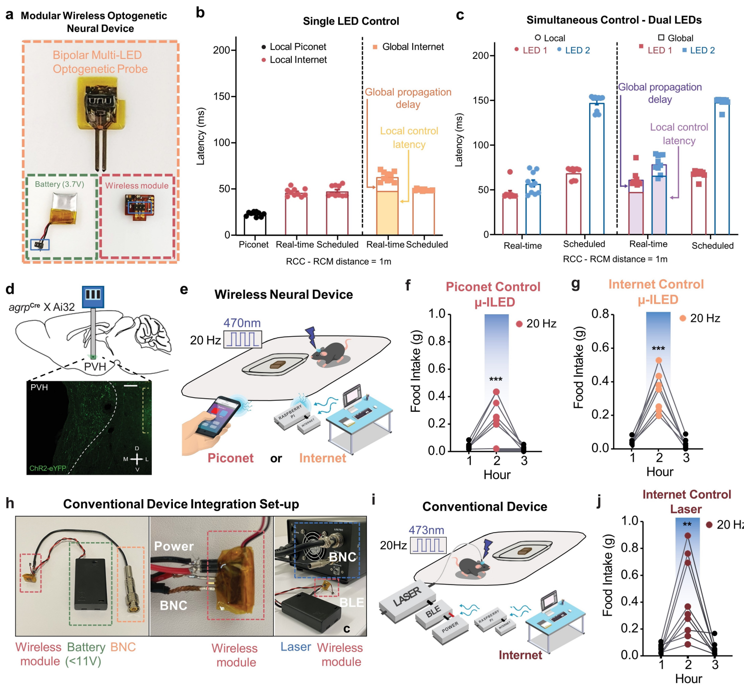
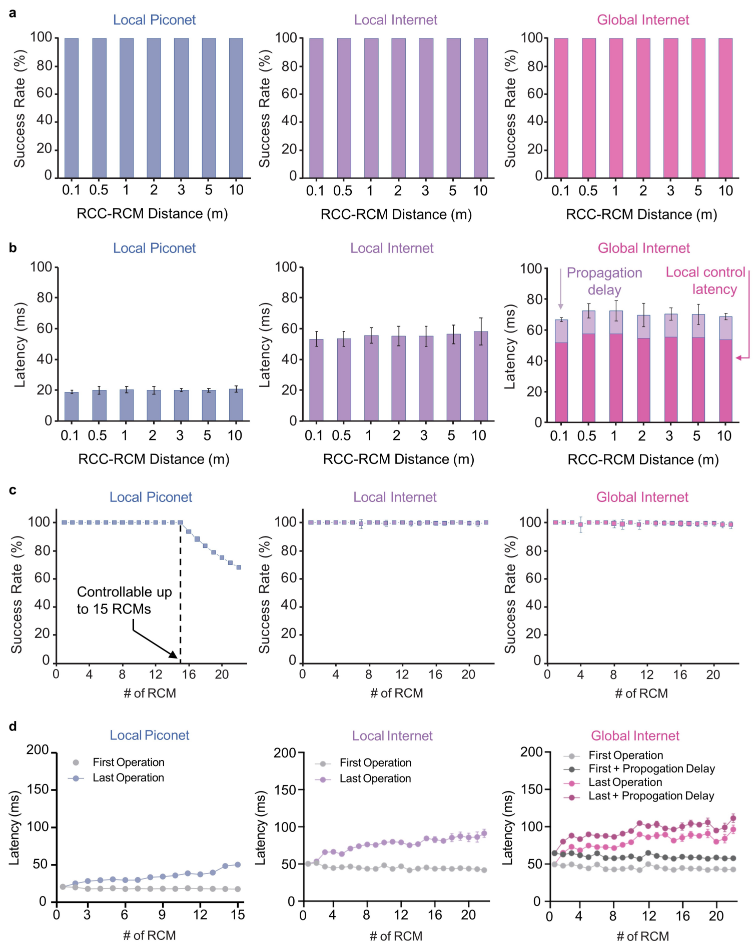


Fig. 3 | Implementation of local and global IoTN network control for *in vivo* neuroscience experimentation. **a**, Three major components of the modular and customizable wireless optogenetic system: a rechargeable LiPo battery, a soft μ-ILED probe and a programmable wireless BLE module (RCM). **b**, Latency of triggering single μ-ILED-based neural probes integrated with wireless RCMs, comparing the responsiveness of local piconet, local and global internet modes (n = 10). Distance between RCC and RCM is 1 m. **c**, Latency of simultaneous triggering of dual μ-ILEDs in optogenetic neural probes integrated with wireless RCMs, comparing the responsiveness of local and global internet modes (n = 10). Orange arrow in **b** and dark purple arrow in **c** indicate a global signal propagation delay, which is dependent on the inter-distance between the remote user and RCC (e.g., 15 ms delay between Boulder, CO and St. Louis, MO, USA). Note that this propagation delay can be ignored in scheduled controls. Distance between RCC and RCM is 1 m. **d**, Sagittal brain cartoon (top panel) of implantation of μ-ILED device into the PVH of *agrp*^{Cre} x Ai32 mice and representative coronal image (bottom panel) showing ChR2-eYFP expression (green) within the PVH. Scale bars, 100 μm. **e**, Cartoon depicting *ad libitum* feeding experiment with either piconet or Internet control. **f-g**, Amount of food consumed during Local Piconet-controlled (**f**), Local internet-controlled (**g**), *agrp*^{Cre} x Ai32 mice consume significantly more food during 20 Hz photostimulation compared to non-stimulation epochs (1 hour) (repeated measures one-way ANOVA, $^{**}p < 0.01$, $^{***}p < 0.001$, n = 7). **h**, Photographs depicting set up of conventional device integration for Internet-controlled conventional optogenetic stimulation. **i**, Cartoon depiction of *ad libitum* feeding experiment using conventional optogenetic stimulation. **j**, Amount of food consumed during internet-controlled conventional optogenetic stimulation, *agrp*^{Cre} x Ai32 mice consume significantly more food during 20 Hz photostimulation compared to non-stimulation epochs (1 hour) (repeated measures one-way ANOVA, $^{**}p < 0.01$, n = 9).

In addition, **Supplementary Fig. 6** and its figure legend has been added as follows:



Supplementary Fig. 6 | Performance characteristics of local and global IoTN network. a-b, Success rate (**a**; n = 20) and latency (**b**; n = 5) of triggering μ-ILED neural probes with wireless RCMs as a function of different RCC-RCM distances (0.1, 0.5, 1, 2, 3, 5, and 10 m), comparing the responsiveness of local piconet, local internet, and global internet modes. The purple-color parts in the global internet latency graph (**b**, right) indicates a signal propagation delay, which is dependent on the inter-distance between the remote user and RCC (e.g., 15 ms delay between Boulder, CO and St. Louis, MO, USA). **c-d,** Success rate (**c**; n = 20) and latency (**d**; n = 5) of triggering a μ-ILED in optogenetic neural probe integrated with wireless RCMs as a function of different numbers of RCMs (1 - 22; 1 m distance from RCC), comparing the responsiveness of local piconet, local internet, and global internet modes. Note that the latency of the global internet control is determined by summing the local control latency and the distance-dependent propagation delay.