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Authors
Liao, Maofu
Cao, Erhu
Julius, David
et al.

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Single particle electron cryo-microscopy of a mammalian ion channel

Maofu Liao[1], **Erhu Cao**[2], **David Julius**[2], and **Yifan Cheng**[1]

[1]Department of Biochemistry and Biophysics, University of California, San Francisco, California 94158-2517, USA

[2]Department of Physiology, University of California, San Francisco, California 94158-2517, USA

Abstract

The transient receptor potential (TRP) ion channel family is large and functionally diverse, second only to potassium channels. Despite their prominence within the animal kingdom, TRP channels have resisted crystallization and structural determination for many years. This barrier was recently broken when the three-dimensional structure of the vanilloid receptor 1 (TRPV1) was determined by single particle electron cryo-microscopy (cryo-EM). Moreover, this is the first example in which the near atomic resolution structure of an integral membrane protein was elucidated by this technique and in a manner not requiring crystals, demonstrating the transformative power of single particle cryo-EM for revealing high-resolution structures of integral membrane proteins, particularly those of mammalian origin. Here we summarize technical advances, in both biochemistry and cryo-EM, that led to this major breakthrough.

Introduction

To date, the vast majority of membrane protein structures, including those representing all major ion channel families, have been determined by X-ray or electron crystallography, necessitating formation of three-dimensional (3D), or two-dimensional (2D) crystals in solution or within lipid bilayers [1,2]. In either case, obtaining well-diffracting crystals is absolutely necessary and remains a major roadblock to success [3]. This is particularly true for mammalian membrane proteins, whose complexity and expression requirements further confound protein crystallization. Transient receptor potential (TRP) channels certainly exemplify such challenges, where long-standing efforts by many groups have failed to produce crystals of sufficient quality for structural analysis.

Single particle electron cryo-microscopy (cryo-EM) circumvents the requirement of well-ordered crystals for structure determination and therefore represents a transformative approach for studying membrane proteins, such as TRP channels, that are refractory to

Corresponding authors: David.Julius@ucsf.edu and ycheng@ucsf.edu.

crystallization. Using this method, proteins in their native conformations are embedded in vitreous ice at liquid nitrogen temperature and imaged directly in the EM. Images of many identical or similar molecules in random orientations are recorded with low electron doses, typically at ~20 $e^{-}/Å^{2}$, to minimize radiation damage of sensitive biological samples. This leads to an extremely low signal-to-noise ratio (SNR) in each individual image, making it necessary to align and average tens of thousands of individual particle images to generate a 3D reconstruction at high resolution. The resolution of 3D reconstruction is improved by iteratively refining geometric orientation parameters for each particle to high accuracy and to correct aberration parameters of each electron micrograph.

In recent years, single particle cryo-EM has achieved near-atomic resolution (~3 Å) for large viral particles with icosahedral symmetry [4], but, analysis of integral membrane proteins was limited to significantly lower resolution of ~ 10Å [5]. However, major technological breakthroughs have now enabled this technique to achieve high resolution for a wider range of protein assemblies, such as the ribosome (~4MDa without symmetry)[6], and proteasome 20S core particle (~700kDa with D7 symmetry) [7]. By exploiting these recent advances in cryo-EM, we recently determined a 3.4Å resolution structure of TRPV1 ion channel without crystals (Fig. 1) [8]. Moreover, we determined the structures of TRPV1 in three distinct conformations, thereby revealing mechanisms of ligand-induced channel activation [9].

These studies showcase the power of single particle cryo-EM for determining structures of integral membrane proteins in multiple conformational states. Four main factors enabled atomic structure determination of TRPV1 without crystals; they include: i) production of high-quality, biochemically stable protein; ii) availability of well characterized pharmacological agents; iii) novel technologies related to direct electron detection camera, including dose fractionation imaging and correction of motion-induced image blurring; and iv) the ability to classify heterogeneous protein conformations. In this review, we summarize these important factors and discuss some unresolved questions and challenges.

Brief overview of TRPV1 biology

TRP channels are found throughout the animal kingdom and are among the most diverse group of ion channels, second only to the potassium channel superfamily. TRP channels resemble voltage-gated potassium channels in regard to overall transmembrane topology and oligomeric structure: they consist of four subunits, each having six transmembrane domains plus a re-entrant pore loop between TM5 and TM6 that constitutes the ion permeation pathway. Cytoplasmic N- and C-terminal domains differ widely among members of this extended ion channel family, with very diverse organization of different protein motifs, such as ankyrin repeat, PDZ, or kinase domains [10,11].

Physiologic roles for many TRP subtypes remain enigmatic, reflecting, in part, a dearth of pharmacological tools that can be used to manipulate these channels *in vivo* or *in vitro*. TRPV1 is a notable exception; as the known site of action for capsaicin and other pungent agents, its contribution to pain sensation has been widely validated through pharmacologic and genetic studies [12]. In addition to serving as a receptor for plant-derived irritants, TRPV1 is also activated by noxious heat (> 43°C) and inflammatory agents, making it an

important player in mechanisms underlying thermosensation, nociception, and pain hypersensitivity. This ability to detect and integrate a wide array of physiologic and environmental signals highlights likely roles for other TRP channels as polymodal signal integrators, making TRPV1 a model for understanding the structural basis for allosteric regulation of TRP channels at large.

Critical factors leading to atomic structure determination of TRPV1

Membrane protein expression, purification, and stabilization

Obtaining high-quality protein samples remains a major challenge in membrane protein structural biology. For eukaryotic membrane proteins, yeast (*Pichia pastoris*), insect (sf9 and Hi 5), and mammalian (HEK293S) cells have been used as hosts for heterologous expression. Mammalian cells can be advantageous because, in some cases, their biosynthetic complexity can better meet lipid or other co-factor requirements of membrane protein targets [13,14]. However, generating milligram quantities of membrane protein typically needed for crystallographic analysis is time consuming, costly, and in some cases impossible if expression proves toxic to the host. Single particle cryo-EM mitigates these problems simply because it requires far less material when compared with crystallographic approaches, on the order of microgram versus milligram quantities. Importantly, this advantage extends to co-factors, toxins, or other natural or synthetic ligands that lock receptors into specific functional states, but which are in precious supply.

To further enhance protein production efforts, we have exploited recently developed BacMam technology as an attractive alternative to standard transfection methods. This system combines advantages of rapid baculovirus propagation in insect cells with highly efficient transduction of mammalian cells and rapid time course (48 hrs) of protein expression [15]. Together, these factors can boost efficiency and robustness of membrane protein expression, especially on the sub-milligram scale commensurate with cryo-EM studies.

In so far as protein stability is concerned, two main factors predominated in our experience with TRPV1. The first involved identification of a so-called ‘minimal subunit’ in which non-essential regions of the protein could be trimmed away without impairing channel function. This enabled us to eliminate especially flexible, unstructured regions within the amino- and carboxy-termini and an extracellular loop – a strategy that is commonly employed for most structural studies, including those involving X-ray crystallography. The second, which is more specific to the cryo-EM approach, involved exchanging detergent solubilized ‘minimal’ TRPV1 into an amphipathic polymer (amphipol A8-35) that maintained solubility and enhanced stability of the purified channel protein. Amphipols stabilize some membrane proteins better than detergents [16], and their use can improve preparation of cryo-EM grids in which membrane proteins are embedded in a thin layer of vitreous ice [17].

Recording high-quality cryo-EM data

In 1995, Richard Henderson theorized that, under perfect sample and imaging conditions, it is possible to achieve 3D reconstructions of ~100kDa molecules to ~3Å resolution from

only 10,000 particle images [18]. Indeed, Henderson's Prediction set the goalpost for technological development in the cryo-EM field. Over the past 15 years, a number of limitations toward achieving this goal have been identified, including suboptimal quality of image recording medium (either photographic film or scintillator based charge-coupled-device (CCD) camera), and image blurring caused by beam-induced motion [19]. Indeed, our best 3D reconstruction of TRPV1 from images obtained with a traditional scintillator based CMOS camera was limited to ~8Å, which is adequate to resolve transmembrane helices, but insufficient for building an accurate atomic model.

A major technological breakthrough came with introduction of a direct electron detection camera [20] and its practical use in recording cryo-EM images of frozen hydrated protein samples [21,22]. These new cameras electrons are detected directly by a silicon sensor, rather than by phosphor scintillators that convert electrons to photons, dramatically improving detection efficiency while greatly reducing background noise. With significantly improved detective quantum efficiency (DQE) at all frequencies, images of frozen hydrated protein samples of relatively small size can now be recorded with a sufficiently small defocus value to retain both high resolution signal and low resolution image contrast [7]. Furthermore, direct detection cameras with a fast frame rate can readout multiple subframes during the exposure (dose fractionation), enabling development of algorithms that correct for beam-induced motion blurring of images obtained from frozen hydrated protein samples [6,7,23].

Our single particle cryo-EM studies of TRPV1 benefited greatly from each of these technological advances [7]. After motion correction, Thon rings of almost all images recorded with the K2 Summit were visible at ~3Å resolution (Fig. 2a), demonstrating marked improvement over images recorded with scintillator based CMOS camera (Fig. 2b). Indeed, 2D class averages of images from the K2 camera showed substantially greater detail when compared with those obtained with a scintillator-based camera (Fig. 2c and d). Furthermore, dose fractionation enabled us to use the best subframes for calculating final 3D reconstruction, a strategy that we established previously using archaeal proteasome 20S core particle as a test specimen [7]. Specifically, we excluded the first two frames, which typically contain too much motion within the subframe, as well as the last 10 frames, in which samples have already suffered severe radiation damage. In the case of TRPV1, this procedure resulted in noticeable improvement of resolution, from 3.6Å to 3.4Å.

Classification of heterogeneous TRPV1 particles

Another major advantage of single particle cryo-EM is the ability to classify images of frozen hydrated protein samples with heterogeneous conformations [6]. In our studies of TRPV1, this played a significant role because, despite all efforts to optimize sample quality and stability, some degree of protein heterogeneity persists. Using a maximum likelihood based 3D classification algorithm implemented in RELION [24], we were able to classify all 45,625 TRPV1 particles into 6 groups (Fig. 3a). Without 3D classification to sort out heterogeneous populations, our 3D reconstruction of TRPV1 had a shape that resembled the correct outline of the channel, but with a hollow interior, particularly within the transmembrane core (TM) (Fig. 3b). Among all six classes, only the 3D reconstruction from

class I (containing ~20% of total particles) showed convincing feature of transmembrane helices. The final 3D reconstruction determined from this data subset was refined to ~8Å resolution (Fig. 3c). This example highlights the importance of sorting out a homogenous population of particles from a heterogeneous dataset.

Some unresolved questions

Refinement of the atomic model

In single particle cryo-EM, the density map is determined independently from atomic model building. After a final density map is determined, it is often low-pass filtered to the estimated resolution and sharpened by a negative temperature factor to enhance high-resolution features of the map. The atomic model is then built into the final low-pass filtered and sharpened density map. At the estimated 3.4Å resolution, the final density map of TRPV1 was sufficient for *ab initio* atomic model building. For this, we used a standard crystallographic model-building tool, Coot [25], and optimized the atomic model locally. However, refining this model globally was more challenging when compared with other structures determined by single particle cryo-EM to similar resolution, such as archaeal 20S proteasome [7], for which a molecular dynamics flexible fitting procedure was used to refine fitting of the atomic structure to the density map [26]. In the case of icosahedral virus particles, refinement was achieved by a pseudo-crystallographic approach using CNS [27]. For TPRV1, neither approach yielded satisfactory results, perhaps owing to non-uniform resolution of different regions of the final density map and/or the presences of densities contributed from amphipols.

There are a number of other approaches or methods that could potentially be used to refine the atomic structure of TRPV1. One is to segment regions of the density map according to their local resolution and refine the atomic structure piece by piece. This is a tedious approach that might or might not yield a reliable result. Another option is to use more sophisticated real space docking refinement procedures. Local resolution variations must be taken into consideration to generate residue specific temperature factors. A procedure based on ROSETTA is being developed for this purpose [28]. It may also be possible to improve refinement of models by increasing agreement to other statistical features of proteins, such as the rotameric enrichment of side chain density peaks using real space sampling methods borrowed from X-ray crystallography [29,30].

Accurate resolution estimation

The resolution of single particle cryo-EM reconstruction is commonly estimated using Fourier Shell Correlation (FSC) procedure [31]. There are also a number of validation methods to make sure that resolution is properly estimated [32]. The estimated resolution of the TRPV1 3D reconstruction is 3.4Å, using the gold-standard Fourier Shell Correlation criteria [31]. However, resolution of the entire 3D reconstruction is not uniform; transmembrane domains (in particular, the pore forming region and conserved TRP domain) are better resolved than cytosolic domains (such as the cytoplasmic tails). There are several methods that estimate local resolution [33,34], but this remains very challenging. We tested our TRPV1 density maps using ResMap, a recently published method [34]. Qualitatively,

the variation of local resolutions in different regions of the density map correlates well with resolved structural features (Fig. 4). Quantitatively, the local resolution of most transmembrane domains was estimated to be ~2.7Å. However, judging from the structural features of these density maps, particularly the shape of side chain densities, the resolution is unlikely to be as high as 2.7Å. Therefore, the information from local resolution estimates should still be viewed with caution.

Symmetry

In all cases of iterative refinement of 3D reconstructions, including those for TRPV1 alone or in complex with ligands, we applied C4 symmetry. We also applied C2 or no symmetry in each of these reconstructions and found some asymmetrical structural features. However, we could not determine whether these are *bona fide* features, or simply attributed to lower signal-to-noise ratios since the resulting 3D reconstructions had both lower resolution and greater noise compared to those calculated with C4 symmetry. Thus, for putative non-symmetrical structural features, it is necessary to include more particle images in the initial analysis so that resolution is not reduced when compared with symmetrized density maps.

Future perspective on using single particle cryo-EM for structural analysis of integral membrane proteins

The TRPV1 structure obtained by cryo-EM is comparable in resolution to that of many other ion channel structures determined by X-ray crystallography [35-37]. Indeed, single particle cryo-EM offers great potential - and certain advantages over X-ray crystallography - for analyzing membrane proteins, particularly those of mammalian origin, in multiple functional or even transient conformations. This is because cryo-EM does not require the formation of well-ordered crystals from large (milligram) amounts of purified protein, and it has the potential to group particle images with even subtle conformational heterogeneities into discrete classes for structural analysis and model building. With further technological developments, it should be possible to improve cryo-EM resolution to better than 3Å. Moreover, integral membrane proteins can be reconstituted into lipid nanodiscs [38] such that their structures can be examined in a more native environment using single particle cryo-EM - an approach that should be superior to current strategies that rely on forming two-dimensional crystals in lipid bilayers.

Our recent success with TRPV1 is extremely encouraging, but numerous challenges lie ahead in making this technique generally applicable to a broad range of integral membrane proteins. Currently, image alignment relies largely on relatively low-resolution information. Thus, achieving atomic resolution of hetero-oligomeric membrane proteins with pseudo symmetry (such as nicotinic acetylcholine receptors or other hetero-pentameric ligand gated channels) would be very challenging. Also, many membrane proteins are ≤100kDa in size and without symmetry. Accurate image alignment of such small particles is also difficult [18]. A potential approach to facilitating studies of such small and/or hetero-oligomeric membrane proteins by single particle cryoEM would be to use monoclonal fragment of antigen binding (Fab) as fiducial markers [39].

Conclusions

The structure of the TRPV1 ion channel is the first atomic structure of an integral membrane protein determined by single particle cryo-EM. This accomplishment is a step closer to the Henderson Prediction and demonstrates the real possibility of determining atomic structures of integral membrane proteins in a way that does not require crystals. Importantly, cryo-EM also stands as an appealing method for determining structures of the same protein in distinct conformations – a goal that is at the very heart of elucidating mechanisms of protein function and pharmacology. Overall, cryo-EM holds promise for still further improvement in resolution as remaining questions and technical challenges continue to be addressed. We hope that lessons learnt from these recent studies will help catapult the application of cryo-EM for structural analysis of other integral membrane proteins, and stimulate further improvements in imaging and analysis methodologies.

Acknowledgments

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Highlights

- Single particle cryo-EM is now capable of determining *ab initio* atomic structures of integral membrane proteins.
- Production of high quality, biochemically stable proteins is a necessity for structural studies.
- Novel technologies related to direct electron detection camera enabled high quality cryo-EM data acquisition
- Ability to classify heterogeneous conformations is essential for achieving atomic resolution.

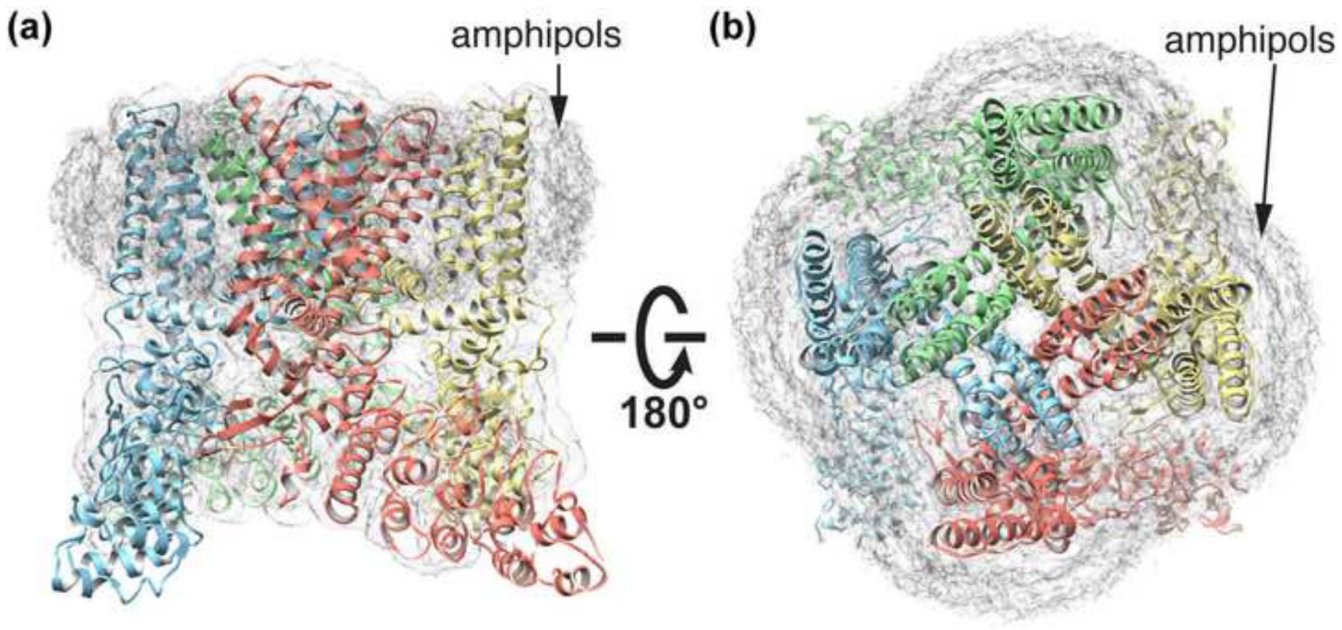

Figure 1. Structure of rat TRPV1 in amphipols

(a) Side and (b) top views of the structure of rat TRPV1 in ribbon diagram. The density of amphipols is marked.

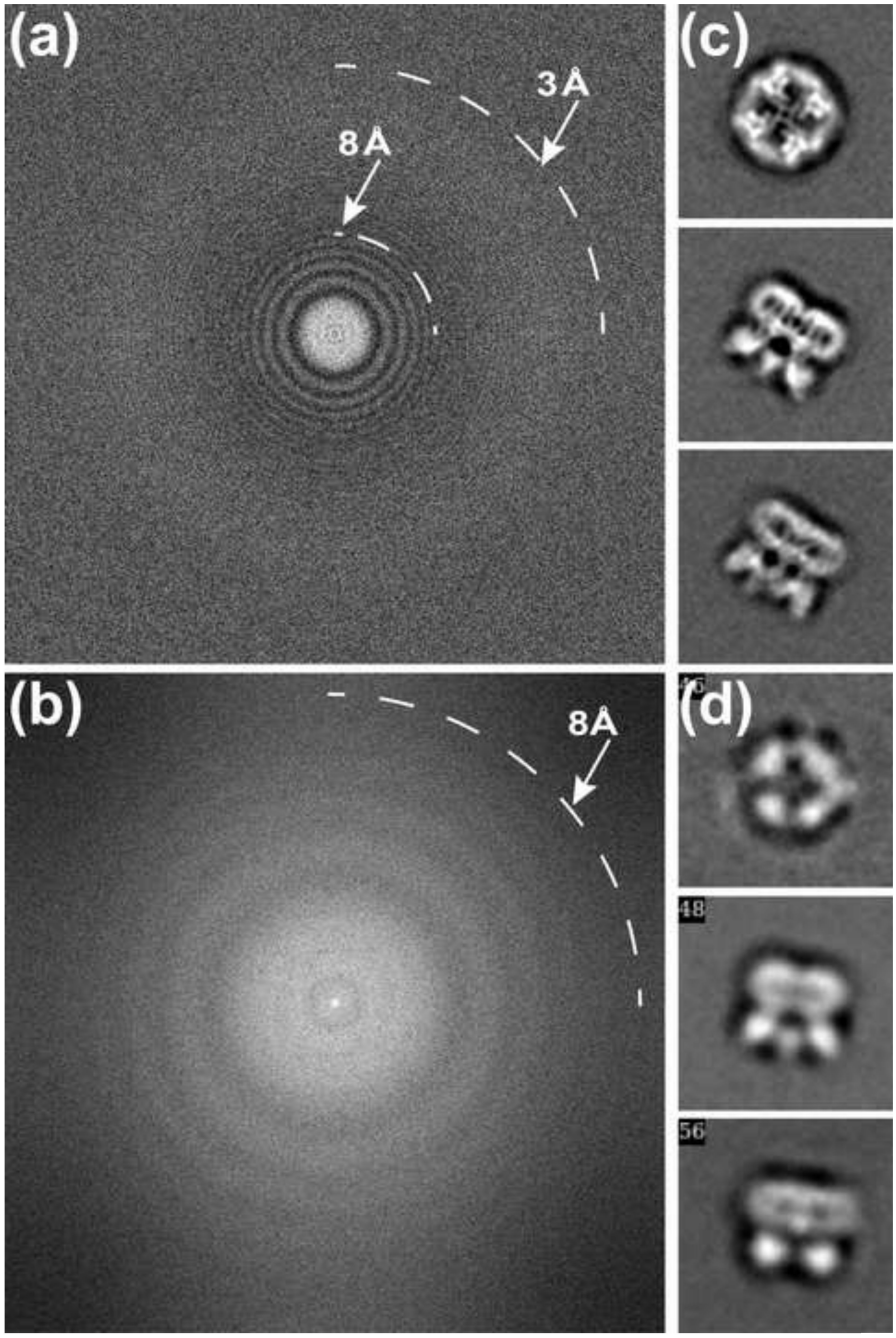

Figure 2. Cryo-EM data are significantly improved with direct detection camera and motion correction

(a) Fourier transform of a typical cryo-EM image of TRPV1 ion channel embedded in thin layer of vitreous ice recorded with the direct detection camera, K2 Summit, after motion correction. Thon rings are visible up to ~3Å resolution. The position corresponding to 8Å is also marked. (b) Fourier transform of a similar image recorded using a phosphor scintillator based CMOS camera, TemF816. Thon rings are visible up to ~8Å resolution. (c) Three typical 2D class averages of images recorded with the K2 camera. (d) Similar 2D class averages of TRPV1 particle images recorded with the TemF816.

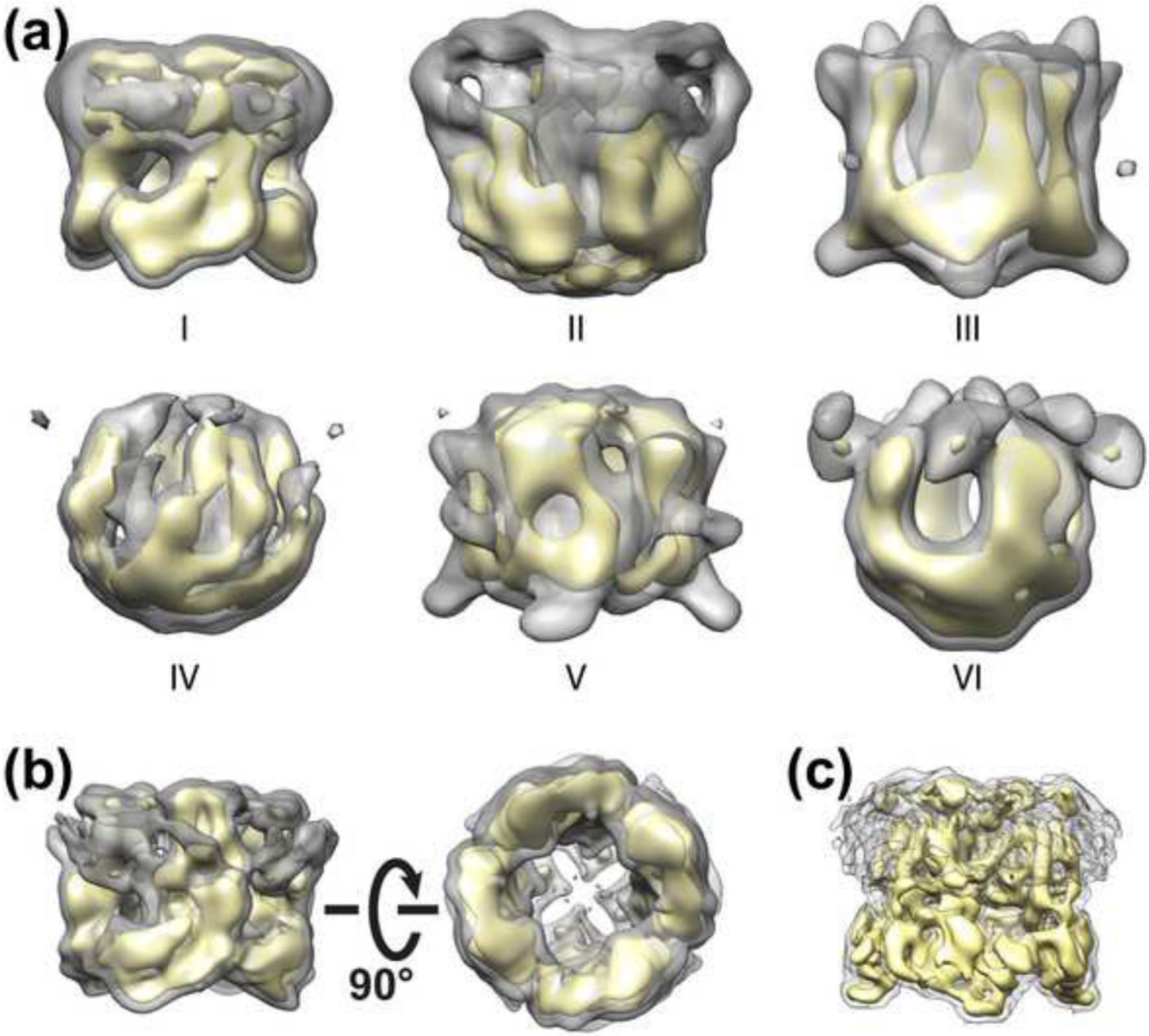

Figure 3. 3D classification of particle images of the TRPV1 ion channel

(a) A total of 45,625 particle images were classified into six classes. 3D reconstructions calculated for each class are shown. The number of particles in each class is: 10,357 (I); 9,527 (II); 3,778 (III); 11,671 (IV); 5,328 (V); 4,964 (VI). Only the first class can be refined to high resolution, 8.8Å. (b) Two different views of the 3D reconstruction calculated from the entire dataset. The nominal resolution is ~9.3Å, but the center of the transmembrane domain is hollow, showing no sign of transmembrane helices. (c) The final 3D reconstruction calculated from class I at a nominal resolution of 8.8Å, showing all transmembrane helices.

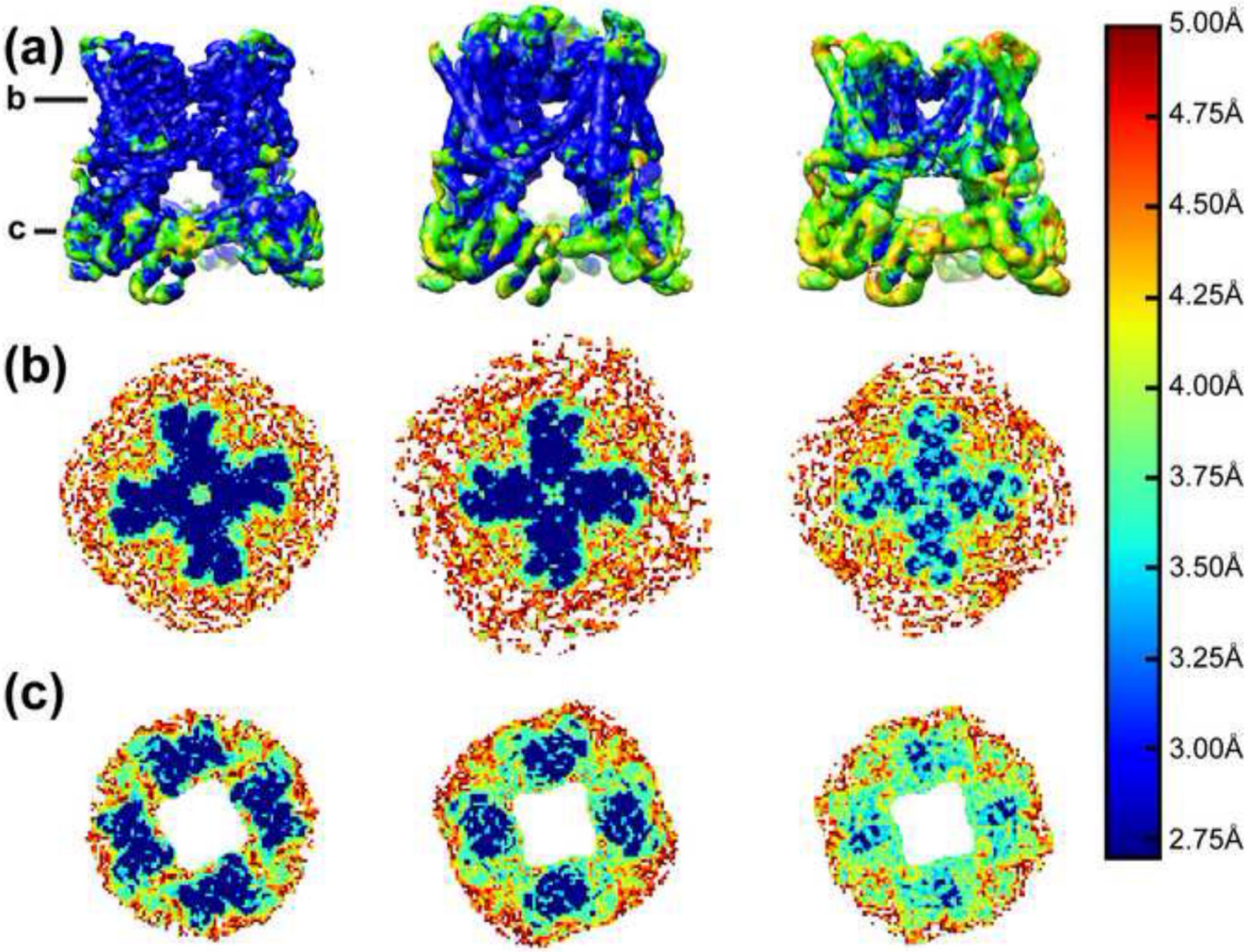

Figure 4. Estimation of local resolution

(a) Local resolution of 3D reconstructions of TRPV1 (left), in complex with RTX/DkTx (middle) and capsaicin (right). Local resolutions are color-coded. The color scale is shown in the right side. (b) and (c) are two slides (marked in (a)) perpendicular to the 4-fold symmetry axis.