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Two-dimensional IR spectroscopy can be designed to eliminate the diagonal peaks and expose only the crosspeaks needed for structure determination

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Contributed by Robin M. Hochstrasser, August 6, 2001

The power of two-dimensional (2D) IR spectroscopy as a structural method with unprecedented time resolution is greatly improved by the introduction of IR polarization conditions that completely eliminate diagonal peaks from the spectra and leave only the crosspeaks needed for structure determination. This approach represents a key step forward in the applications of 2D IR to proteins, peptides, and other complex molecules where crosspeaks are often obscured by diagonal peaks. The technique is verified on proteins, peptides, and other complex molecules where crosspeaks are eliminated by diagonal peaks from the spectra and leave only the crosspeaks needed for structure determination. The introduction of IR polarization conditions completely eliminates the diagonal peaks from the 2D IR spectra.

Table 1. Some polarization conditions for 2D-IR spectroscopy

<table>
<thead>
<tr>
<th>α*</th>
<th>β</th>
<th>γ</th>
<th>δ</th>
<th>Tensor element†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>π/4</td>
<td>−π/4</td>
<td>0</td>
<td>1/2 (xyxy) + (yxxy)</td>
</tr>
<tr>
<td>π/4</td>
<td>0</td>
<td>−π/4</td>
<td>0</td>
<td>1/2 (xyxy) + (yxxy)</td>
</tr>
<tr>
<td>π/4</td>
<td>−π/4</td>
<td>0</td>
<td>0</td>
<td>1/2 (xyxy) + (yxxy)</td>
</tr>
<tr>
<td>π/3</td>
<td>−π/3</td>
<td>0</td>
<td>0</td>
<td>1/4 (xxxx) − 3(xyxy)</td>
</tr>
<tr>
<td>0</td>
<td>π/3</td>
<td>−π/3</td>
<td>0</td>
<td>1/4 (xxxx) − 3(xyxy)</td>
</tr>
<tr>
<td>θ</td>
<td>−θ</td>
<td>π/2</td>
<td>0</td>
<td>1/2 sin2θ (xyxy) − (yxxy)</td>
</tr>
<tr>
<td>π/2</td>
<td>θ</td>
<td>−θ</td>
<td>0</td>
<td>1/2 sin2θ (xyxy) − (yxxy)</td>
</tr>
<tr>
<td>circ</td>
<td>circ</td>
<td>0</td>
<td>0</td>
<td>1/2 (xxxx) + (yxxy)</td>
</tr>
<tr>
<td>circ</td>
<td>0</td>
<td>circ</td>
<td>0</td>
<td>1/2 (xxxx) + (yxxy)</td>
</tr>
</tbody>
</table>

*The angles measured with respect to one of the polarization axes; circ means circularly polarized light of a given sense, and results are obtained from laboratory fixed field unit vectors that are complex. For example, for a process generating a macroscopic polarization \( P_0 \), the general tensor element would be \( \langle \epsilon^{\alpha \beta \gamma \delta} \rangle \).
†Subscripts have been removed, e.g. \( \langle xxyy \rangle = \langle \epsilon^{\alpha \beta \gamma \delta} \rangle \), because the relations are true for all paths i,j,k,l. In our experiment, \( x \) and \( y \) are orthogonal polarization axes perpendicular to the direction of pulse propagation, z.

gathered about the coupling between different spatial regions of the molecule and about the dynamics of the structural changes for these regions.

We have chosen two simple systems to demonstrate this approach. The first is 1,3-cyclohexanedione, which has two C=O groups giving symmetric and antisymmetric stretching bands in the linear IR spectrum, but whose coupling is not known. This molecule thus serves as a model system to develop the method. The second system is acetylproline-NH2, which has been studied previously with heterodyned 2D IR spectroscopy (3, 4). Acetylproline-NH2 is a dipeptide and also has two C=O groups that give rise to two amide I bands and one observable amide II band. The dipeptide was found to adopt two different conformations in CDCl3, and its 2D IR spectrum has been assigned. We show here that the structural information content of the 2D IR spectrum is significantly enhanced with the information gained from the polarization measurements.

Materials and Methods

The laser system developed to perform the 2D IR experiments consists of an amplified titanium sapphire laser that pumps a regenerative dye laser (7). The dye laser consists of an amplified titanium sapphire laser that pumps a regenerative dye laser (7). The laser system developed to perform the 2D IR experiments consists of an amplified titanium sapphire laser that pumps a regenerative dye laser (7). The laser system developed to perform the 2D IR experiments consists of an amplified titanium sapphire laser that pumps a regenerative dye laser (7). The laser system developed to perform the 2D IR experiments consists of an amplified titanium sapphire laser that pumps a regenerative dye laser (7).
sample, where it is overlapped and heterodyned with the emitted photon echo signal in the phase matching direction \( k_{LO} = -k_1 + k_2 + k_3 \). The pulses \( k_1 \) and \( k_2 \) are separated by time \( \tau \), \( k_1 \) and \( k_3 \) are separated by \( T \) that is set to zero for a two-pulse echo, and \( k_{LO} \) arrives a time \( t \) after \( k_1 \) and \( k_2 \). The heterodyned photon echo is collected as a function of \( \tau \) and \( t \). For the 1,3-cyclohexane-dione experiments, \( \tau \) and \( t \) were stepped from 0 to 2,500 fs, and for acetylproline-NH\(_2\), \( \tau \) and \( t \) were stepped from 0 to 1,800 fs. The \( \tau \) and \( t \) signals were undersampled by collecting data in 18-fs increments (the fundamental period is \( \approx 10 \) fs). The resulting spectra are aliased and corrected by addition of the spectral width (3). The polarizations of all of the pulses are controlled with wire-grid polarizers (Specac; 12900) placed before the sample; a polarizer after the sample selects the polarization of the photon echo. Three polarization conditions are used in this report: (0, 0, 0, 0), (0, 0, 0), (0, 0, 0, 0), (0, 0, 0, 0). The pulses follow in the experiment \((k_1, k_2, k_3, k_{LO})\), and \( 0 = \) vertical. The samples are about 100 mM in CDCl\(_3\) and are held between two CaF\(_2\) windows that are spaced 50 \( \mu \)m apart. The (0, 0, 0, 0, 0) measurement of acetylproline-NH\(_2\) is consistent with previously published spectra (3), although the signal strength is improved by a factor of 10.

The 1,3-cyclohexane-dione spectrum taken with \((\pi/4, -\pi/4, \pi/2, 0)\) polarization (see Fig. 1b below) consists of eight peaks whose peak positions are determined by the frequencies and anharmonicities (see Eq. 2 below). It is a straightforward task to determine these parameters for well-separated peaks (5), but when the separations are less than the linewidths, as is the case here, an accurate fit requires a known lineshape (2). The lineshape is calculated from the dephasing time, \( T_2 \), and the inhomogeneous broadening, \( \sigma \). These parameters were determined from two-pulse photon echo measurements and a 2D IR spectrum by using \((0, 0, 0, 0)\) polarization and gave \( T_2 = 575 \pm 50 \) fs and \( \sigma = 7 \) cm\(^{-1}\). To determine the frequencies and anharmonicities, the \((\pi/4, -\pi/4, \pi/2, 0)\) spectrum was least-squares fit to eight peaks. The parameters were constrained to reproduce the frequencies in the linear spectrum,\(^7\) and the intensities and signs of the peaks in the 2D IR spectrum were allowed to vary. The resulting frequencies and anharmonicities are listed below.

After the incident pulse, an echo field, analogous to the free induction decay of NMR, is emitted that is combined with the local oscillator pulse on a detector to give the measured signal \( S_{\text{abgd}}(\tau; T) \) as a function of the time intervals; the subscripts follow the polarization condition \((\alpha, \beta, \gamma, \delta)\). The 2D IR spectrum is generated from the time-domain data by calculating the 2D Fourier transform of the measured signal,

\[
S_{\text{abgd}}(\omega_1, \omega_2; T) = e^{i\phi} \int_0^\infty e^{i(\omega_1 \tau + \omega_2 T)} \{ S_{\text{abgd}}(\tau, T)h(t)dt \} d\tau \tag{1}
\]

where \( h(t) \) is a window function, and \( \phi \) is a phase factor, described previously (2). \( S_{\text{abgd}}(\omega_1, \omega_2; T) \), hereafter written \( S[\alpha\beta\gamma\delta] \), is a complex function, and in this paper the magnitude \( |S[\alpha\beta\gamma\delta]| \) and the real spectrum \( \text{Re}\{S[\alpha\beta\gamma\delta]\} \) are reported.

**Results**

**1,3-Cyclohexane-dione.** The linear-IR spectrum for 1,3-cyclohexane-dione in CDCl\(_3\) has two peaks located at 1,711 and 1,735 cm\(^{-1}\), which are the symmetric and antisymmetric C=O stretch modes. The ratio of their areas is \( \approx 2:1 \). Fig. 1a shows \( \text{Re}\{S[0, 0, 0, 0]\} \), and peaks contribute to the 2D spectra, labeled Q through Z. The two peaks in the linear spectrum each produce a positive peak on the diagonal, labeled U and W, and a negative peak that is shifted to lower frequencies along \( \omega_0 \), labeled T and V. The pattern that each linear peak produces a pair of out-of-phase peaks near the diagonal is typical for 2D IR spectra (2). Peaks R/S and X/Y are crosspeaks that correlate the diagonal peaks to one another, and each pair of crosspeaks consists of one positive and one negative peak, similar to the diagonal pairs. However, the signs of the crosspeaks are reversed for this molecule as compared with the diagonal peaks; the lower-frequency peaks R and X are positive, and the higher-frequency peaks S and Y are negative. This phase reversal is because the angle between the transition dipoles of the symmetric and antisymmetric stretch is 90°, which is larger than the magic angle (54.7°) (3, 12). Peaks Q and Z arise from transitions that are expected to be very weak. Some peaks are not so evident in Fig. 1a, but they are more apparent in \( \text{Re}\{S[0, 0, 0, 0]\} \), shown in Fig. 1b. The relative intensities of the peaks are dramatically different in the \( \text{Re}\{S[\pi/2, -\pi/2, 0, 0]\} \) spectrum. Peaks U and W have been eliminated in this spectrum, and peaks T and V are only a few percent of their original intensity. Crosspeaks R/S and X/Y are now the dominant features. Peaks Q and Z are also more apparent.

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*The entire spectrum was shifted \(-2 \text{ cm}^{-1}\) along \( \omega_1 \) and \( \omega_2 \) to calibrate the frequency axes. The origin of the calibration is discussed in ref. 2.*
Acetylproline-NH₂. The linear-IR spectrum of acetylproline-NH₂ in CDCl₃ has three peaks that arise from the amide I and II modes (3). The three peaks each produce a pair of peaks along the diagonal similar to 1,3-cyclohexanedione, which are observed in Re{S[0,0,0,0]} as previously reported (3). Because the pairs are split by less than their linewidths, they appear as single peaks in Abs{S[0,0,0,0]}, shown in Fig. 2a. The diagonal peaks are labeled A, B, and C in Fig. 2a. Peaks A and C are from the amide II and I vibrational modes located on the amino end of the molecule. Peak B is the amide I band on the acetyl end. Six crosspeaks are observed above the diagonal and labeled D through I. Each of these crosspeaks couples the amide I and II frequencies. From previous polarization measurements (3), the angles between the transition dipoles for all of the crosspeaks are known.

Fig. 2 shows Abs{S[1,2,0,0], −F[1,2,0,0]}, where the diagonal peaks A, B, and C are suppressed by about 90–95%. The crosspeaks are now clearly visible on the lower half of the spectrum as well. The peaks in Fig. 2b are labeled J, K, L, and M. Clearly J and K are a set of crosspeaks, as are L and M. It will be shown below that J results from crosspeaks D and E, and L from F and G. Re{S[1,2,0,0]} is shown in Fig. 2c. The out-of-phase pairs of peaks are now clearly visible for each of the crosspeaks in Fig. 2a. However, the phase of the pairs is not the same for all of the peaks. The set of peaks that form J and K have the same phase on either side of the diagonal, but L and M are out-of-phase.

Analysis and Discussion. First, we account for the peak positions in the 2D IR spectra and predict their relative contributions and signs depending on the polarizations of the pulses. The 1,3-cyclohexanedione spectra are then fit to determine the frequencies and anharmonicities. In the final section, the peak intensities observed in the {S[1,2,0,0]} spectrum for acetylproline-NH₂ are explained with previously measured transition dipole angles, and a simulation is presented that accounts for the phases of the observed crosspeaks.

2D IR Signals. In principle, all of the vibrational states of a molecule having vibrational quantum numbers 1 and 2 can be accessed in a third-order photon echo experiment. As a result, the fundamentals, overtones, and combination bands all contribute. For a single vibrator, the two- or three-pulse echo involves a fundamental and an overtone state (2). For a system with N oscillators, there will be N one-quantum states and N(N + 1)/2 states that have the excitations on different oscillators (combination modes). The vibrational energy for a two-mode system up to second order in the vibrational quantum numbers \( \nu_1 \) and \( \nu_2 \) is

\[
G(\nu_1, \nu_2) = \omega_1 (\nu_1 + \frac{1}{2}) + \omega_2 (\nu_2 + \frac{1}{2})^2 + \omega_1 \left( \nu_1 + \frac{1}{2} \right) + \omega_2 \left( \nu_2 + \frac{1}{2} \right)^2 + \omega_1 (\nu_1 + \frac{1}{2}) + \omega_2 (\nu_2 + \frac{1}{2}) \left( \nu_1 + \frac{1}{2} \right) \]

where \( \omega_1 \) and \( \omega_2 \) are the frequencies of the fundamentals and are the anharmonicities. The shifts between the \( \nu = 0 \) to 1 \( \chi_{ab} \) and their \( \nu = 1 \) to 2 transitions are \( \Delta_\alpha = -2\chi_{ab} \), and the off-diagonal anharmonicity is \( \Delta_\beta = \chi_{ab} \). For the 1,3-cyclohexanedione system, the one-quantum states are the symmetric and antisymmetric stretching modes of the molecule. For the acetylproline-NH₂ system, the
Polarization Effects. In this section, we introduce a number of polarization configurations to select the pathways, \( \mathcal{P} \). Each contribution to the third-order nonlinear response depends on a fourth-rank tensor that describes the orientation of the four-molecule fixed transition dipole moments defining the path, \( i,j,k, \) and \( l \), and the laboratory fixed set of polarization directions \( \alpha, \beta, \gamma, \) and \( \delta \). A typical factor is \( (\alpha_0(0)\beta_0(\tau + T)\delta(\tau + T + t)) = (\alpha_0\beta_0\delta_\tau) \), where the time arguments are implicit in the last expression. From known properties of polar fourth-rank tensors in isotropic media, the general tensor element is the product of two factors, one that depends on the polarizations of the exciting fields and another that includes only the time-dependent relative orientations of the dipoles (12).

In isotropic media, only three independent macroscopic observables are obtainable from variations in the polarizations of the incident fields (14). If we choose \( x \) and \( y \) as any two of the three orthogonal directions in the lab frame, the following condition is maintained between the nonvanishing tensor elements for each of the paths:

\[
\langle x, y, x, y \rangle = \langle x, y, y, x \rangle + \langle x, y, y, y \rangle + \langle x, y, y, y \rangle
\]  

Note that there are no terms having only one \( x \) or one \( y \). From these general relations, it is straightforward to write down the specific tensor elements that are measured in any chosen polarization condition. Some useful examples are given in Table 1, showing that different linear combinations of the tensor elements can be obtained by judicious choices of polarizations of the four pulses. Depending on the specification of the molecular dipole axes, some of these linear combinations may exist, and others may not. Therefore the polarization conditions may be used to examine specific microscopic contributions to the 2D IR spectrum. Such procedures have been used in many other forms of nonlinear spectroscopy, such as in coherent anti-Stokes Raman and Raman-induced Kerr effect measurements (15).

The fourth to seventh entries in Table 1 are particularly useful in 2D IR, because the signals vanish for certain pathways involved in the interaction of a molecule with IR pulses. We consider a molecule having two vibrations, 1 and 2, and hence two possible transition dipole directions in the molecule frame. These could correspond to excitations of the two amide I groups in acetylproline-NH\(_2\), or the symmetric and asymmetric stretches of 1,3-cyclohexanediol. The polarization condition \( (\pi/4, -\pi/4, \pi/2, 0) \), which maximizes the signal given in the sixth row of Table 1, allows the measurement of the tensor combination \( \langle x_3y_3 z_3 y_3 \rangle \) for which the various possible paths is given by:

\[
\begin{align*}
\langle x_1, y_1, x_1, y_1 \rangle &- \langle x_1, y_1, y_1, x_1 \rangle = 0 \tag{5a} \\
\langle x_1, y_1, y_2, x_1 \rangle & - \langle x_1, y_2, x_1, y_1 \rangle = f(t, T, t)(1 - P_2) \tag{5b} \\
\langle x_1, y_1, y_2, x_2 \rangle & - \langle x_1, y_2, x_2, y_1 \rangle = -f(t, T, t)(1 - P_2) \tag{5c} \\
\langle x_1, y_1, x_2, y_2 \rangle & - \langle x_1, y_2, x_2, y_2 \rangle = 0 \tag{5d}
\end{align*}
\]

where \( P_2 = 3(\cos^2\theta_{12} - 1) \), and \( \theta_{12} \) is the angle between the transition dipoles and the average is over any fixed distribution.
of structures. The internal motions and overall rotations are considered to be independent. All of the terms in Eq. 5 vanish when the transition dipoles are parallel ($P_2 = 1$). The diagonal contributions to the 2D IR spectrum vanish (Eq. 5a), whereas the crosspeaks in the spectra will remain whenever $P_2 \neq 1$ (Eq. 5b and c). When the molecules are not rotating during the experiment, which is commonly the case when the coherence dynamics are faster than the orientational relaxation time, we find $f = 5/9$. The values of $f(t, T, t)$ depend on the specific models for the rotational dynamics (16). Eq. 5a was recently proven for a symmetric diffuser even with the transition dipole not parallel to a principal diffusion axis (17). The values of $f(t, T, t)$ for a spherical rotor are tabulated in ref. 12. If the transition dipoles are parallel to principal diffusion axes, such as with 1,3-cyclohexanedione, the calculations are greatly simplified and yield the inequalities in Eq. 5 by straightforward application of known principles (16). The ensemble averages needed to obtain $f(t, T, t)$ in the case of asymmetric rotors are functions of the diffusion of the molecule about its principal axes but can be calculated easily by standard methods for all possible cases (16).

The ($\pi/4, -\pi/4, \pi/2, 0$) polarization condition from line 6 of Table 1 completely removes peaks arising from diagonal regions when internal motions are neglected. Thus, the signal from the first line of Eq. 3 should be completely eliminated from 2D IR spectra by using ($\pi/4, -\pi/4, \pi/2, 0$) polarization. Hence peak b is removed from the spectra in Fig. 3, and peak a should be greatly diminished because only the forbidden term remains.

**Simulation of the Polarized 2D IR Spectrum of 1,3-Cyclohexanedione.** The origin of the peaks in the 1,3-cyclohexanedione spectra is immediately apparent from Fig. 3; all 10 peaks predicted by Eq. 3 are present in Re{$S\tilde{\omega}[0, \pi/4, \pi/4, 0]$} of Fig. 1, Peaks T/U and V/W correspond to peaks a and b in Fig. 3, peaks R/S and X/Y correspond to peaks c and d, and peaks Q and Z correspond to peak e. As predicted above, the ($\pi/4, -\pi/4, \pi/2, 0$) measurement does indeed remove peaks arising from parallel transitions, as evidenced by the elimination of peaks U and W and the suppression of peaks T and V in Fig. 1b.

The peak positions are determined by the one- and two-quantum eigenstate energies, which need to be extracted from the 2D IR spectra to determine the frequencies and anharmonicities in Eq. 2. The simulated spectrum is shown in Fig. 4 a–c, which models the data extremely well, and the resulting frequencies and anharmonicities are $\omega_1 = 1,730.2$, $\omega_2 = 1,746.5$, $\Delta_1 = 13.1$, $\Delta_2 = 5.5$, $\Delta_{12} = 12.4$ cm$^{-1}$.
The question of how to provide the frequencies and anharmonicities with a structural basis remains. In peptides and proteins, the C==O stretches, or the amide I modes, are mechanically localized on each peptide unit and only weakly perturbed from their zero-order energies by electrostatic coupling to amide I modes (11). In such a picture, the zero-order modes must convert into the eigenmodes of the whole system by a simple perturbation calculation within each nearly degenerate group. If the calculation of the eigenmodes were to require coupling among zero-order modes of different types, the exciton model would be wrong. This appears to be the case for 1,3-cyclohexanediene, whose spectra are not predicted by a simple exciton model. The construction of its symmetric and antisymmetric stretch modes must require the participation of other modes, so there is not a simple approximation in which the C==O excitations are localized and from which the eigenmodes are retrieved by a perturbation calculation. Thus, the C==O modes are determined by the motions of the entire molecule, and the force constant matrix needs to be diagonalized to ascertain their energies. The frequencies and anharmonicities are still sensitive to the structure of the molecule, but a simple electrostatic coupling model based on isolated modes, such as used for peptides, does not apply for 1,3-cyclohexanediene. The 2D IR and linear IR measurements on peptides and 1,3-cyclohexanediene support the aforementioned differences.

Simulation of the 2D IR Spectrum of Acetylproline-NH$_2$. The ($\pi/4$, $-\pi/4$, $\pi/2$, 0) polarization experiment in Fig. 2b substantially improves the spectral discernability of the crosspeaks. The relative crosspeak intensities also change according to Eq. 5 because of their differing angular dependencies (3). For instance, crosspeak F, which is very weak in the Abs {0[0,0,0]} spectra of Fig. 2a, has significant intensity in Fig. 2b as peak L, because the two transition dipoles involved are almost at 90°. Crosspeak G in Fig. 2a also contributes to peak L (see below). The transition dipoles that form peak H, which is not very intense in Fig. 2b, are at 35°. The signal corresponding to peak I is not visible in Fig. 2b, because the transition dipoles involved are nearly parallel ($\phi = 1$).

The phases of the crosspeaks also help to distinguish the different conformers. For example, peaks L and M are about 180° out-of-phase with each other in Fig. 2c, whereas peaks J and K are in-phase. Fig. 5 simulates two distributions that could arise between the transition dipoles of amide I modes (11). In such a picture, the zero-order modes must convert into the eigenmodes of the whole system by a simple perturbation calculation within each nearly degenerate group. If the calculation of the eigenmodes were to require coupling among zero-order modes of different types, the exciton model would be wrong. This appears to be the case for 1,3-cyclohexanediene, whose spectra are not predicted by a simple exciton model. The construction of its symmetric and antisymmetric stretch modes must require the participation of other modes, so there is not a simple approximation in which the C==O excitations are localized and from which the eigenmodes are retrieved by a perturbation calculation. Thus, the C==O modes are determined by the motions of the entire molecule, and the force constant matrix needs to be diagonalized to ascertain their energies. The frequencies and anharmonicities are still sensitive to the structure of the molecule, but a simple electrostatic coupling model based on isolated modes, such as used for peptides, does not apply for 1,3-cyclohexanediene. The 2D IR and linear IR measurements on peptides and 1,3-cyclohexanediene support the aforementioned differences.

The Use of Polarized IR Pulses in Structure Determination. The structural information of 2D IR comes from the angular and coupling information. The angles between transition dipoles in peptides have been measured by polarization in double resonance 2D IR and heterodyned 2D IR experiments (3, 6, 18). Usually two separate measurements are compared: one where the laser pulses have the same polarization, and another where they do not. In heterodyned and double resonance 2D IR experiments, the measurements in the two polarization conditions can be subtracted to yield difference spectra with the diagonal peaks removed (3, 6), if the molecule does not rotate. In practice, there is always some overall or internal rotation, so the method contains intrinsic uncertainties. Furthermore, it can be difficult to normalize the two spectra recorded in different polarizations, especially when the crosspeaks and diagonal peaks overlap. These difference spectra can be measured directly by using ($\pi/3$, $-\pi/3$, 0, 0) and ($\pi/3$, 0, $-\pi/3$, 0) polarizations (Table 1), without the need to normalize or collect multiple spectra, but the diagonal peaks are eliminated for spectra collected by using ($\pi/4$, $-\pi/4$, $\pi$, 0), even if the molecule rotates; rotation simply diminishes the signal. Thus the shapes and positions of the crosspeaks are made much clearer. The next step of analyzing the peaks to understand how the pieces of the structure move with respect to one another (4) is now possible.

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